

# Open Research Online

---

The Open University's repository of research publications and other research outputs

## The effect of elevated CO<sub>2</sub> on nitrogen allocation between components of the photosynthetic machinery in Spring wheat

### Thesis

#### How to cite:

Theobald, Julian C. (2001). The effect of elevated CO<sub>2</sub> on nitrogen allocation between components of the photosynthetic machinery in Spring wheat. PhD thesis The Open University.

For guidance on citations see [FAQs](#).

© 2000 The Author



<https://creativecommons.org/licenses/by-nc-nd/4.0/>

Version: Version of Record

Link(s) to article on publisher's website:  
<http://dx.doi.org/doi:10.21954/ou.ro.0000e2e4>

---

Copyright and Moral Rights for the articles on this site are retained by the individual authors and/or other copyright owners. For more information on Open Research Online's data [policy](#) on reuse of materials please consult the policies page.

---

[oro.open.ac.uk](http://oro.open.ac.uk)

UNRESTRICTED

# **The Effect of Elevated CO<sub>2</sub> on Nitrogen Allocation between Components of the Photosynthetic Machinery in Spring Wheat**

Julian C. Theobald, B.Sc. (Hons) University of Wales, Bangor  
M.Sc. University of Wales, Aberystwyth

IACR-Rothamsted, Harpenden, UK  
Open University, UK

May 2000

Submitted for the degree of Ph.D in Plant Physiology/Biochemistry

AUTHOR NO: P927686X

DATE OF SUBMISSION: 22 MAY 2000

DATE OF AWARD: 01 AUGUST 2001

**3<sup>RD</sup> PARTY MATERIAL  
EXCLUDED FROM  
DIGITISED THESIS**

**PLEASE REFER TO THE  
ORIGINAL TEXT TO SEE  
THIS MATERIAL**

## NOTIFICATION OF REDACTION

**THESIS TITLE:**

The effect of elevated CO<sub>2</sub> on Nitrogen allocation between components of the photosynthetic machinery in Spring Wheat

**AUTHOR:**

Julian Theobald

**YEAR:**

2000

**CLASSMARK:**

633.1189 THE

The following pages/sections have been redacted from this thesis:

Page No.	Item/section redacted
4	Matrial after Appendix A on pg 311 (3 <sup>rd</sup> party articles)



## **Additional Statement**

All experimental work was the responsibility of Julian Theobald who was assisted by others in its execution. The work was planned under the supervision of myself and results discussed with me by Julian. The procedure for the analysis of gas-exchange response data in Chapter 6 was developed by me.

Dr. R.A.C. Mitchell

I hereby declare that this thesis has not been submitted, either in the same or different form, to this or any other University for a degree.

Signature:

## ACKNOWLEDGEMENTS

This work was funded by a grant from the European Union as part of the European Stress Physiology and Climate Experiment (ESPACE)-Wheat project (contract no. EV5V-C793-0301). All the research work was done at IACR-Rothamsted, Harpenden, Hertfordshire, which receives grant-aided support from the Biotechnology and Biological Sciences Research Council of the United Kingdom. Writing of the thesis progressed in my 'spare' time, surrounded by the green and lush fells of Lancashire, and was funded by my 'other' full-time job!

I am indebted to my supervisor at Rothamsted, Dr Rowan Mitchell for his patience and generosity of time, advice, and encouragement over many months. I hope that many others follow to benefit from his guidance. I would also like to thank Drs David Lawlor, Martin Parry and John Andralojc for their interest and encouragement, and particularly the latter for his advice regarding the ATP-synthase work, and his unrelenting sharp wit and good humour! Thanks also go to Ms Valerie Mitchell and Mr Simon Driscoll for technical assistance, and to all former colleagues of the Biochemistry and Physiology Department at Rothamsted who helped in one way or another.

The kind gift of antisera against CF<sub>1</sub> from Professor J.C. Gray (University of Cambridge, UK) is gratefully acknowledged. Other acknowledgements are made in the text, where appropriate.

I am grateful to family and friends for their support, and particularly my parents for an inspiring rural upbringing, which unquestionably is responsible for my curiosity and interest in the way that the natural world around me works. To my wife of two months, Anne; this PhD is as much yours as it is mine – what more can I say!

Finally, I wish to dedicate this thesis to the memories of family and friends, who always supported, showed interest, and gave encouragement to my endeavours.

Over Kellet, Lancashire, June 2001

## ABSTRACT

Wheat (*Triticum aestivum* L. cv Minaret) was grown long-term under CO<sub>2</sub> partial pressures of 36 and 70 or 100 Pa with various N applications (4 to 23 g m<sup>-2</sup> N), to test hypotheses of N re-allocation: 1) a decrease in N from leaves to other organs, 2) a relative decrease in N from Rubisco to other photosynthetic components.

Elevated CO<sub>2</sub> did not affect phenology, mainstem leaf appearance, the pattern of N allocation throughout the plant, or the fraction of crop N in grain at harvest, but 1) stimulated biomass and yield by 5 to 20% over the N range used, and 2) caused a faster loss of N and components from flag leaves during grain-fill.

Responses of photosynthesis to varying  $pC_i$  were fitted, and rates of maximal carboxylation and non-photorespiratory respiration estimated. The former, was proportional to Rubisco content, and light-saturated photosynthetic rate at 70 Pa CO<sub>2</sub> was proportional to ATP-synthase. Potential photosynthetic rates at 70 Pa CO<sub>2</sub> were calculated, compared with observed, and used to estimate excess investment in Rubisco. The excess was greater in high N treatments than low, declining as leaves senesced. The fraction of Rubisco estimated to be in excess, was strongly dependent on leaf N content, increasing from ~5% in leaves with 1 g N m<sup>-2</sup> to ~40% in leaves with 2 g N m<sup>-2</sup>. Growth at elevated CO<sub>2</sub> usually decreased the excess somewhat, but only as a consequence of a general decrease in leaf N, given that relationships of components to leaf N content were independent of CO<sub>2</sub> and N treatment, demonstrating that no direct CO<sub>2</sub> effect on N allocation within leaves had occurred. It is concluded that there is scope for improving the N-use efficiency of C<sub>3</sub> crop plants in elevated CO<sub>2</sub> conditions, by genetic manipulation to decrease the amount of Rubisco.

Title Page	
Additional Statement	
Declaration	i
Acknowledgements	ii
Abstract	iii
Contents	iv
List of Figures	ix
List of Abbreviations	xii

## CHAPTER 1

### General Introduction

1.1	Overview	1
1.2	Nitrogen: Relationship to photosynthesis and patterns of allocation	4
1.3	The steady-state biochemical model of C <sub>3</sub> photosynthesis	9
1.4	Evidence for the C <sub>3</sub> model and the co-limitation of capacities	11
1.4.1	Short-term response of photosynthesis and the activity of components to elevated $pC_a$	11
1.4.2	Evidence from transgenic plants	15
1.4.3	Evidence from long-term changes in amounts of components in response to altered irradiance and temperature	19
1.5	Long-term responses to elevated $pC_a$	21
1.5.1	Biomass and yield	21
1.5.2	Stomatal conductance and water use	21
1.5.3	Photosynthesis	22
1.5.3.1	Mechanisms of photosynthetic acclimation	23
1.5.3.2	Theoretical responses for N optimisation within the photosynthetic machinery	26
1.6	Evidence for acclimation and N optimisation within the photosynthetic machinery in response to elevated $pC_a$	30
1.7	Aims and Objectives	34

## CHAPTER 2

### Methodology for environmental treatments, growth of plants, routine techniques, and effect of elevated partial pressures of CO<sub>2</sub> on plant growth and yield

2.1	CO <sub>2</sub> exposure facility	37
2.2	Plant Growth	40
2.3	Nutrient applications	44
2.4	Chlorophyll concentration	46
2.5	Soluble protein concentration	46
2.6	Polyacrylamide Gel Electrophoresis (PAGE)	47
2.6.1	Gradient gel casting	48
2.6.2	Preparation of Samples for PAGE	49

2.6.3	Electrophoretic separation of proteins	50
2.6.4	Coomassie staining	50
2.7	Tissue N concentration	51
2.7.1	Sample preparation	51
2.7.2	Determination by instantaneous combustion	51
2.8	Preliminary experiment to determine the effects of variable N supplies on aspects of growth, photosynthesis and leaf biochemistry in spring wheat grown at 36 Pa CO <sub>2</sub>	52
2.8.1	Introduction	52
2.8.2	Materials and Method	53
2.8.3	Results and discussion	55
2.9	Determination of extraneous nitrate-N and ammonium-N content in Terra-green/Perlite rooting medium	60
2.9.1	Introduction	60
2.9.2	Materials and method	60
2.9.3	Results and discussion	61
2.10	The effects of elevated partial pressures of CO <sub>2</sub> on plant growth and yield	62
2.10.1	Introduction	62
2.10.2	Materials and Method	62
2.10.3	Results and discussion	64

### CHAPTER 3

#### The long-term effect of elevated partial pressures of CO<sub>2</sub> on the *in vivo* balance between capacities determining photosynthesis in wheat

3.1	Introduction	81
3.2	Materials and Methods	84
3.2.1	Calculation of photosynthetic parameters	88
3.2.2	Experiment to demonstrate the light saturation of $A/pC_i$ responses	89
3.2.3	Experiment to determine whether photosynthetic rates at high $pC_i$ decline through the photoperiod in wheat	91
3.2.4	Criteria for photosynthesis measurements in Experiments One to Four	94
3.3	Results: Responses of leaf N content, parameters derived from $A/pC_i$ curves and the O <sub>2</sub> sensitivity of photosynthesis to long-term growth at 70 Pa CO <sub>2</sub> as a function of time	95
3.4	Results: Responses of parameters derived from $A/pC_i$ curves and the O <sub>2</sub> sensitivity of photosynthesis to long-term growth at 70 Pa CO <sub>2</sub> as a function of leaf N content	119
3.5	The effect of CO <sub>2</sub> enrichment on the depression of photosynthesis through the photoperiod	127
3.6	The effect of CO <sub>2</sub> enrichment on the accumulation of soluble carbohydrate in flag leaves of spring wheat	130
3.7	Conclusions	135

## CHAPTER 4

### The long-term effect of elevated partial pressures of CO<sub>2</sub> on the amount and activation of Rubisco in Spring Wheat

4.1	Introduction	139
4.2	Development of an Extraction Protocol for Rubisco	141
4.2.1	The effect of centrifugation time on Rubisco recovery	141
4.2.2	The effect of protease inhibitors, PVP, length of standing on ice and sample re-freezing on the recovery of Rubisco from samples	145
4.2.3	The effect of Triton-X-100 on the recovery of Rubisco	148
4.2.4	Summary	151
4.3	Quantification of Rubisco by ELISA	151
4.3.1	Introduction	151
4.3.2	Materials and method	152
4.3.3	Comparison of different Rubisco standards	157
4.3.4	Section summary	159
4.4	[ <sup>14</sup> C]CABP binding assay for the quantification of Rubisco	160
4.4.1	Introduction	160
4.4.2	Preparation of [ <sup>14</sup> C]CABP	160
4.4.3	Sample preparation	161
4.4.4	[ <sup>14</sup> C]CABP Assay	161
4.4.5	Calculations	162
4.4.6	Preliminary testing of the [ <sup>14</sup> C]CABP binding assay	162
4.5	Measurement of Rubisco initial, total and maximal activities	165
4.5.1	Introduction	165
4.5.2	Materials and method	166
4.5.3	Preliminary assessment	167
4.6	The effect of elevated partial pressures of CO <sub>2</sub> on the amounts of leaf components	170
4.6.1	Introduction	170
4.6.2	Results - Amount of Rubisco in leaves as a function of time	171
4.6.3	Discussion	173
4.6.4	Results - amounts of Chlorophyll, soluble protein and Rubisco as a proportion of total leaf	175
4.6.5	Discussion	182
4.7	The effect of elevated CO <sub>2</sub> on the activity of Rubisco	184
4.7.1	Introduction	184
4.7.2	Results - Activity of Rubisco as a function of time	185
4.7.3	Results - Activity of Rubisco as a function of leaf N content	187
4.7.4	Discussion	188
4.8	Conclusions	191

## CHAPTER 5

### The long-term effect of elevated partial pressures of CO<sub>2</sub> on the amount of Coupling Factor 1

5.1	Introduction	193
5.2	Determination of Mg <sup>2+</sup> -specific ATP-ase activity	195
5.2.1	Basic method for determining the Mg <sup>2+</sup> specific ATP-ase activity in wheat	196
5.2.2	The effect on CF <sub>1</sub> hydrolytic activity, of standing extracted samples on ice for prolonged periods prior to assay	200
5.2.3	The effect of assay incubation temperature and duration on CF <sub>1</sub> hydrolytic activity	201
5.2.4	The effect of spiking assayed samples	203
5.2.5	Pea versus Wheat: a comparison of CF <sub>1</sub> hydrolytic activity	206
5.2.6	Sample extraction procedure: A comparison between current method applied to wheat, and that used by Evans (1987) for pea	208
5.2.7	The effect on CF <sub>1</sub> hydrolytic activity of longer homogenisation, filtration, and shortening of the thylakoid centrifugation step	209
5.2.8	Experiment to confirm the reproducibility of the improved activity assay	211
5.2.9	Discussion	212
5.3	Quantification of Coupling Factor 1	214
5.3.1	Isolation and purification of the ATP-ase coupling factor (CF <sub>1</sub> ) from wheat, for use as a standard	215
5.3.2	Quantification of standards and samples by electrophoresis, Western blotting and Enhanced Chemi-Luminescence (ECL)	221
5.3.3	Experiments to determine whether CF <sub>1</sub> and Rubisco could be accurately quantified from a single extraction of each leaf sample	228
5.4	The effect of long-term growth at 70 Pa CO <sub>2</sub> on the amount of coupling factor (CF <sub>1</sub> )	235
5.4.1	Introduction	235
5.4.2	Results - amount of CF <sub>1</sub> in leaves as a function of time	235
5.4.3	Discussion	238
5.4.4	Results - amounts of Chl, soluble protein and CF <sub>1</sub> as a proportion of total leaf N - effects of elevated CO <sub>2</sub>	239
5.4.5	Discussion	247
5.5	Conclusions	250

## CHAPTER 6

### The effect of elevated partial pressures of CO<sub>2</sub> on the relationship between Rubisco and the thylakoid ATP-synthase, their relationships with parameters of photosynthesis, and estimation of the excess investment in Rubisco

6.1	Introduction	253
6.2	The relationship between Rubisco and the thylakoid ATP-synthase	254
6.2.1	Introduction	254



6.2.2	Results	254
6.2.3	Discussion	255
6.3	Relationships between Rubisco, the thylakoid ATP-synthase, and photosynthetic parameters	256
6.3.1	Introduction	256
6.3.2	Method	257
6.3.3	Results	259
6.3.4	Discussion	261
6.4	Estimation of excess Rubisco in leaves grown at 70 Pa CO <sub>2</sub>	262
6.4.1	Method	262
6.4.2	Results	263
6.4.3	Discussion	266
6.5	Conclusions	268
6.6	Critical appraisal of methodology and results	270
6.6.1	Growth environment	270
6.6.2	Whole plant, physiological and biochemical responses	274
6.6.3	Challenges for the future	283
6.7	Summary of results	286
6.7.1	Whole plant responses to long-term growth at elevated $pC_a$	286
6.7.2	Response of the <i>in vivo</i> balance of capacities determining photosynthetic rate to long-term growth at elevated $pC_a$ , determined from gas exchange analysis	287
6.7.3	Response of components of the photosynthetic machinery to long-term growth at elevated $pC_a$	289
6.8	Study Conclusions	291

<b>LITERATURE CITED</b>	293
-------------------------	-----

## APPENDIX

A	Publications arising from this study	311
---	--------------------------------------	-----

Figure 1.1	Relationship between Rubisco and leaf N content.	7
Figure 1.2	Modelled response of photosynthesis to intercellular CO <sub>2</sub> partial pressure ( $pC_i$ ).	12
Figure 1.3	The simulated response of light saturated rates of leaf CO <sub>2</sub> uptake ( $A$ ) with intercellular CO <sub>2</sub> partial pressure ( $pC_i$ ).	29
Figure 2.1	The patterns of application through time, of different N treatments for Experiments One to Four.	44
Figure 2.2	The patterns of N application through time, as d relative to anthesis.	54
Figure 2.3	Assimilation rate versus intercellular CO <sub>2</sub> pressure ( $pC_i$ ) for flag leaves of wheat grown at a range of N applications, and measured between full expansion and anthesis.	57
Figure 2.4	Amounts of Rubisco, Chl and Chl $a/b$ ratio versus soluble protein content.	58
Figure 2.5	Mainstem leaf number through development.	65
Figure 2.6	Number of shoots through development.	66
Figure 2.7	Total crop dry mass, including roots, through time.	68
Figure 2.8	Allocation of total crop dry mass.	70
Figure 2.9	Water soluble carbohydrate content in leaves and stems measured at anthesis.	74
Figure 2.10	Nitrogen applied and total crop N uptake over time.	75
Figure 2.11	Nitrogen allocation to different plant parts.	76
Figure 3.1	Response of net CO <sub>2</sub> assimilation ( $A$ ) to PPFD, in fully expanded flag leaves of spring wheat grown at high N application and different CO <sub>2</sub> partial pressures.	90
Figure 3.2	The effect of a period of photosynthesis through the day under growth conditions, on the $A/pC_i$ response in flag leaves of wheat.	93
Figure 3.3	Total leaf N content versus time in leaves of spring wheat grown at either 36 or 70 Pa CO <sub>2</sub> .	97
Figure 3.4	Carboxylation efficiency versus time in leaves of spring wheat grown at either 36 or 70 Pa CO <sub>2</sub> .	98
Figure 3.5	The maximum rate of net photosynthesis under saturating light and CO <sub>2</sub> ( $A_{max}$ ) versus time as days relative to anthesis.	102
Figure 3.6	CO <sub>2</sub> stimulation ( $A_{70}:A_{36}$ ) versus time.	106
Figure 3.7	Ratio of the change in photosynthetic rate on decreasing the [O <sub>2</sub> ] from 21 to 2kPa.	108
Figure 3.8	Carboxylation efficiency versus total leaf N content.	121
Figure 3.9	$A_{max}$ versus total leaf N content.	122
Figure 3.10	CO <sub>2</sub> stimulation ( $A_{70}:A_{36}$ ) versus total leaf N content.	124
Figure 3.11	Ratio of the change in photosynthetic rate on decreasing the [O <sub>2</sub> ] from 21 to 2kPa versus leaf N content.	125
Figure 3.12	The effect of different measurement times in the photoperiod on the $A/pC_i$ response in flag leaves.	128

Figure 3.13	The response of various carbohydrate concentrations through time, or during the photoperiod, to long-term growth at either 36 or 100 Pa CO <sub>2</sub> in low N grown flag leaves of wheat.	134
Figure 4.1	The effect of increased centrifugation on the recovery of components from leaf material.	144
Figure 4.2	The effect of protease inhibitors on the recovery of Rubisco	147
Figure 4.3	The amount of Rubisco estimated by ELISA versus total leaf N content of flag leaves.	157
Figure 4.4	The relationship between Rubisco and N content in samples measured by both ELISA and [ <sup>14</sup> C]CABP-binding.	163
Figure 4.5	Estimates of the <i>in vitro</i> Rubisco activity versus Rubisco content in flag leaves of wheat.	168
Figure 4.6	The Rubisco content of leaves versus time.	171
Figure 4.7	Chlorophyll versus total leaf N content.	175
Figure 4.8	The chlorophyll a/b ratio versus total leaf N content.	177
Figure 4.9	Soluble protein versus total leaf N content.	178
Figure 4.10	Amount of Rubisco versus total leaf N content.	179
Figure 4.11	Rubisco N as a percentage of total leaf N versus total leaf N content.	181
Figure 4.12	The response through time of the <i>in vitro</i> initial, total and maximal activities of Rubisco.	186
Figure 4.13	Estimates of <i>in vitro</i> Rubisco activity versus Rubisco content.	188
Figure 5.1	The response of wheat CF <sub>1</sub> activity to incubation temperature.	202
Figure 5.2	Relationship between CF <sub>1</sub> activity and thylakoid concentration.	213
Figure 5.3	Protein elution profile.	218
Figure 5.4	SDS polyacrylamide gel electrophoresis of wheat CF <sub>1</sub> .	219
Figure 5.5	Densitometric analysis of wheat CF <sub>1</sub> protein band.	221
Figure 5.6	Autoradiograph from Western blot for quantifying CF <sub>1</sub> .	225
Figure 5.7	Calibration curve for quantification of CF <sub>1</sub> .	226
Figure 5.8	Correlation for different assay of same CF <sub>1</sub> sample.	227
Figure 5.9	Autoradiograph showing response of antiserum against CF <sub>1</sub> .	231
Figure 5.10	Autoradiograph image showing response of antiserum against Rubisco.	233
Figure 5.11	Leaf CF <sub>1</sub> content as a function of time.	236
Figure 5.12	Amount of Chlorophyll verses leaf N.	240
Figure 5.13	Chlorophyll a/b verses leaf N.	241
Figure 5.14	Soluble protein verses leaf N.	242
Figure 5.15	CF <sub>1</sub> verses leaf N.	244
Figure 5.16	Percentage N in CF <sub>1</sub> verses leaf N.	245
Figure 5.17	CF <sub>1</sub> verses Chlorophyll.	246

<b>Figure 6.1</b>	Relationship between ATP-synthase and Rubisco measured in the same leaf sample.	255
<b>Figure 6.2</b>	Rate of estimated $V_{\text{cmax}}$ versus Rubisco content.	260
<b>Figure 6.3</b>	Rate of estimated photosynthesis gross of dark respiration versus ATP-synthase content.	261
<b>Figure 6.4</b>	The estimated excess of Rubisco at 70 Pa $\text{CO}_2$ .	264
<b>Figure 6.5</b>	Estimates of <i>in vivo</i> $V_{\text{cmax}}$ required to maintain observed photosynthetic rates.	265
<b>Figure 6.6</b>	The fraction of Rubisco estimated to be in excess of that required to maintain photosynthetic rate at 70 Pa $\text{CO}_2$ .	267

## List of abbreviations

<i>A</i>	net CO <sub>2</sub> assimilation rate
<i>A</i> <sub>max</sub>	maximum light-saturated CO <sub>2</sub> assimilation rate
ADP	adenosine-5'-diphosphate
ANOVA	analysis of variance
APS	ammonium persulphate
ATP	adenosine-5'-triphosphate
<i>atpD</i>	thylakoid ATP-synthase D subunit gene
BSA	bovine serum albumin
C	carbon
C <sub>3</sub>	first photosynthetic product has three carbon atoms
CA	carbonic anhydrase
<i>cab</i>	chlorophyll a binding protein
Chl	chlorophyll
C <sub>R</sub>	flux control coefficient for Rubisco
d	day
DMSO	dimethyl sulphoxide
DW	dry weight
$\epsilon$	carboxylation efficiency
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FACE	free-air CO <sub>2</sub> enrichment
FBPase	fructose-1,6-bisphosphatase
$\Gamma$	CO <sub>2</sub> photocompensation point
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
<i>g</i> <sub>m</sub>	mesophyll conductance
<i>g</i> <sub>s</sub>	stomatal conductance
IRGA	infra-red gas analyser
KBq	kilo bequerel
LAI	leaf area index
LHC	light harvesting complex
LRB	lower reservoir buffer
LSU	large subunit
N	nitrogen
NADPH	nicotinamide adenine dinucleotide phosphate
OTC	open top chamber
Pa	Pascal
PAGE	polyacrylamide gel electrophoresis
PAR	photosynthetically active radiation
<i>pC</i> <sub>a</sub>	ambient CO <sub>2</sub> partial pressure
<i>pC</i> <sub>c</sub>	chloroplastic CO <sub>2</sub> partial pressure
<i>pC</i> <sub>i</sub>	intercellular CO <sub>2</sub> partial pressure
PCO	photosynthetic carbon oxidation
PCR	photosynthetic carbon reduction
PGA	(D)-3-phosphoglyceric acid
PMSF	phenyl methylsulphonyl fluoride
PNUE	photosynthetic nitrogen use efficiency
PO <sub>4</sub> <sup>3-</sup>	inorganic phosphate

PPFD	photosynthetic photon flux density
PRK	phosphoribulokinase
PVP	polyvinylpyrrolidone
<i>rbcL</i>	Rubisco large subunit gene
<i>rbcS</i>	Rubisco small subunit gene
$R_d$	non-photorespiratory respiration occurring in light
RNA	ribonucleic acid
Rubisco	Ribulose-1,5-bisphosphate carboxylase/oxygenase
RuBP	Ribulose-1,5-bisphosphate
SDS	sodium dodecyl sulphate
SE	standard error
SED	standard error of difference of means
SPS	sucrose phosphate synthase
SSU	small subunit
TEMED	N,N,N',N'-tetramethylethylene-diamine
URB	upper reservoir buffer
$V_{cmax}$	maximum CO <sub>2</sub> and RuBP saturated Rubisco activity
$W_c$	carboxylation capacity
$W_j$	RuBP-regeneration capacity
$W_p$	triose phosphate utilisation capacity

# CHAPTER 1

## General Introduction

### 1.1 Overview

Within the constraints of genetically determined upper limits, the productivity of crop and other plant species depends primarily on how efficiently light energy is used to drive carbon (C) and nitrogen (N) assimilation, and how effectively the products of this primary assimilation are allocated and utilised during growth and development (Quick and Foyer, 1997). Nitrogen, an essential nutrient necessary for building proteins, nucleic acids and other cellular constituents, is often limiting in ecological and agricultural environments. As C and N are integral components for virtually all metabolic functions it follows that if the supply of either is limited, then so too will be overall growth.

If either the N or C supply is severely limiting to growth, there are a number of plant mechanisms which confer flexibility for the alteration of growth patterns and the control of processes, so that the best use of resources and maximal growth are attained for a given environment. At the whole plant level for example, this is achieved through the maintenance of a balance between C assimilation in 'source' leaves and utilisation in 'sinks', the latter being all sites where synthesis and growth takes place. Evidence for this can be observed in the maintenance of characteristic shoot and root dry matter distribution ratios through development (Rufty, 1997). These reflect the need for a functional equilibrium or balance between the processes controlling C and N acquisition, in that the provision of C assimilate from shoots to the root controls the rate of root growth and capacity for N uptake, which is supplied to the shoot and determines C fixation by controlling the rate of shoot

growth and the associated expansion of photosynthetic capacity. Thus in general, increasing N limitation and decreasing C limitation will induce a decrease in the investment in photosynthetic tissue and an increase in roots.

Apart from such morphological shifts, optimisation of N use within photosynthetic tissue is given by maximisation of photosynthesis for a given N content. This involves optimising the investment in the components of photosynthesis, the largest one of which is the primary carboxylating enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco: reasons for which are discussed in section 1.2). There is good evidence that investment of N into photosynthetic components can often be re-balanced and optimised in response to long-term (hours to days) changes in certain environmental parameters, e.g. irradiance (Leong and Anderson, 1984; Evans, 1987) and temperature (Makino et al., 1994). Plants have evolved to deal with fluctuations in these environmental variables. However, plants are now additionally being subjected to a change in an environmental variable which has remained relatively stable over the last 160,000 years (Barnola et al., 1987), namely the atmospheric CO<sub>2</sub> partial pressure ( $pC_a$ ).

The partial pressure of atmospheric CO<sub>2</sub> is increasing annually by as much as 0.18 Pa (Watson et al., 1992), in response to changes in global land use and greater emissions from the burning of fossil fuels since the industrial revolution (Graves and Reavey, 1996). Consequently, current  $pC_a$  is expected to double from 36 to 70 Pa within the next 70 to 90 years. In the short-term (minutes to hours), CO<sub>2</sub> enrichment stimulates photosynthesis and increases the amount of assimilate available for growth. This occurs for two reasons, (1) photorespiration is partially suppressed because CO<sub>2</sub> is a competitive inhibitor of the oxygenation of ribulose-1,5-bisphosphate (RuBP), and consequently there is an increased efficiency of net



photosynthesis with respect to light, water and N use (Long, 1991; Long and Drake, 1992), and (2) current  $pC_a$  is insufficient to saturate Rubisco, thus a further increase in carboxylation velocity can result by increased substrate binding (Webber et al., 1994). However, in the long-term this initial stimulation of photosynthesis has often been observed to decline (Sage et al., 1989; Yelle et al., 1989; Arp 1991), in a response termed photosynthetic acclimation. Thus for plants limited in their growth by such factors, the initial short-term stimulation of photosynthesis by elevated  $CO_2$  seems to decline because the stimulation in supply of carbohydrates exceeds demand from sinks, thereby creating an imbalance within the whole plant. In attempting to redress this imbalance, the amounts of Rubisco and other photosynthetic components have often been observed to decrease (Yelle et al., 1989; Besford, 1990; Rowland-Bamford et al., 1991; Woodrow, 1994). By decreasing the amount of photosynthetic machinery, the N-use efficiency of the plant is enhanced as valuable N removed from the photosynthetic machinery is made available for further growth and utilisation of the extra carbohydrate (Stitt, 1991; Bowes, 1993).

Irrespective of whether a source-sink imbalance occurs within the whole plant upon long-term exposure to elevated  $CO_2$ , which for plants growing under a high nutrient supply in field conditions is typically not the case (Arp and Drake, 1991; Lawlor and Mitchell 1991; Delgado et al, 1994; Nie et al., 1995a), an imbalance will nevertheless arise between the component capacities which determine photosynthetic rate (discussed in section 1.3). This is because at elevated  $pC_a$  Rubisco typically does not limit photosynthesis and is often deactivated (Sage et al., 1988), whilst RuBP-regeneration and possibly phosphate ( $PO_4^{3-}$ )-regeneration do limit the photosynthetic rate. The deactivation of Rubisco suggests that the amount of this enzyme in leaves could be excessive of requirements under such conditions.

With a doubling of  $pC_a$ , C acquisition is unlikely to be a limitation to growth, so that in many environments the major limitation to productivity could shift and reside with the availability of N for the growth of new sinks. For optimal N-use efficiency at 25°C and double the existing  $pC_a$  to 70 Pa, models suggests that the amount of Rubisco could be decreased by 30 to 40% before it would exert an equivalent limitation to that which it imposes for the same leaf at current  $pC_a$  (Webber et al., 1994; Medlyn, 1996). Theoretically, such excess N could be re-allocated to rate-limiting components of the photosynthetic machinery, and/or throughout the plant to generate new sinks.

Thus, in the remainder of this chapter, I shall consider in more detail the patterns of N allocation and the implications of this upon photosynthetic capacity in leaves of  $C_3$  plants, based on the biochemical model of leaf photosynthesis developed by Farquhar et al., (1980). This will act as essential background information from which theories of how N allocation may be altered at various levels within the plant, can be developed. Evidence supportive or critical of such models will be discussed in sections covering both the short and long-term effects of growth at elevated  $CO_2$  on plant responses, both at the physiological and biochemical levels. The chapter will conclude with a summary of the hypotheses that were tested in the work presented in this thesis.

## **1.2 Nitrogen: Relationship to photosynthesis and patterns of allocation**

The relationship between photosynthesis and leaf N content is one of the most important to plant function, since photosynthesis provides the energy and C necessary for reproduction and growth, whilst N availability determines the amount of photosynthetic machinery present, and therefore potential photosynthetic

capacity. As photosynthesis is the major biochemical process occurring in leaves, N invested into photosynthetic carbon reduction (PCR) and thylakoid proteins represents the majority of leaf N. Consequently, the photosynthetic capacity of a leaf is strongly related to its leaf N content, although the gain of this relationship varies between species (Evans and Seeman, 1989). For example, for the same and typical leaf N content of  $1.4 \text{ g m}^{-2}$ , Evans (1989, Fig.1) observed 10-fold differences in net  $\text{CO}_2$  assimilation rates ( $A$ ) across a selection of species, ranging from crops to trees and shrubs inhabiting harsh environments. Wheat (Evans, 1983; 1985) and rice (Cook and Evans, 1983) typically achieved the highest rates of  $A$  at around  $28 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ , with others achieving rates as low as 2 to  $3 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ , so demonstrating considerable variation in photosynthetic N-use efficiency (PNUE; the ratio of the maximal  $A$  under saturating light at the growth  $pC_a$  ( $A_{\text{max}}$ ) for a given leaf N content ( $A_{\text{max}}:\text{N}$ )).

In considering how growth at elevated  $pC_a$  might alter the allocation of N between components of the photosynthetic machinery in leaves and thereby increase PNUE, it is first necessary to consider the allocation of N into these same components at current  $pC_a$ . Nitrogen associated with the photosynthetic machinery accounts for between 55 to 60% of total leaf N in a 'typical'  $\text{C}_3$  sun leaf (Evans and Seeman, 1989). Distribution is between two main pools representing, (1) proteins bound or closely associated with the thylakoid membranes which govern the photosynthetic light reactions and so include those responsible for light harvesting, electron transport and photophosphorylation, and (2) soluble proteins associated with the PCR cycle including those involved in  $\text{CO}_2$  assimilation, RuBP-regeneration, photorespiration and starch and sucrose synthesis. The distribution of N between these pools and their components are summarised in Table 1.1 below.

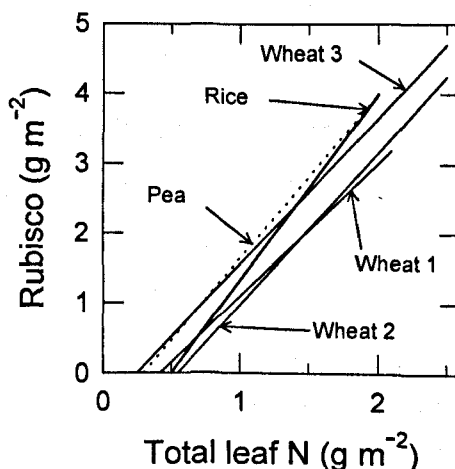
**Table 1.1** Percentage allocation of protein N in a leaf of a 'typical' C<sub>3</sub> sun plant. LHC = Light harvesting complex. Adapted from Evans and Seeman (1989).

<b>Thylakoid Proteins</b>	Light Harvesting – 17.4%	Photosystem II	5.0%
		LHC	6.5%
		Photosystem I	5.9%
	Bioenergetics – 7.2%	ATPase (CF <sub>1</sub> CF <sub>0</sub> )	5.4%
		Electron transport	1.8%
<b>Soluble Proteins</b>	CO <sub>2</sub> fixation – 30.6%	Rubisco	23.0%
		PCR enzymes	6.6%
		Carbonic anhydrase	1.0%
<b>Other Constituents</b>	Biosynthesis – 21.4%	Ribosomes	7.1%
		Nucleic acids	8.5%
		Amino acids	2.5%
		Envelope	3.3%
	Remainder – 23.4%	Other soluble protein	13.3%
		Other	10.1%

From Table 1.1 it is clear that a large amount of leaf N (23%) is allocated solely to Rubisco. This is because Rubisco has a very low specific activity, typically no greater than 1  $\mu\text{mol CO}_2$  fixed  $\text{min}^{-1} \text{mg}^{-1}$  (Leegood, 1993), which is partly due to its large size (550000 Da), of which 15% by mass is N (Evans and Seeman, 1984). Further, as the full name suggests, Rubisco catalyses both the carboxylation and oxygenation of RuBP, with oxygen (O<sub>2</sub>) being a competitive inhibitor of RuBP carboxylation. Uptake of O<sub>2</sub> via the oxygenation of RuBP results in carbohydrate anabolism via the photosynthetic carbon oxidation (PCO) cycle, with the resultant release of CO<sub>2</sub> in photorespiration. Under current  $pC_a$ , the oxygenation of RuBP results in a loss of some 30% of previously fixed C (Long, 1991). Therefore, to support high net photosynthetic rates and compensate for the low specific activity and loss of C to photorespiration, large amounts of Rubisco are required, at a high cost to an often limiting N resource.

Such an abundance of Rubisco protein in leaves results in a strong correlation between the enzyme and total leaf N (Fig. 1.1) in many species.

Generally, and for the examples of wheat, rice and pea illustrated below, straight lines cross the x-axis at positive values of total leaf N, demonstrating that the proportion of total leaf N allocated to Rubisco is not constant, but increases with increasing leaf N content (Evans, 1989).



**Figure 1.1** Relationship between Rubisco and leaf N content. Wheat 1 to 3 (respectively, Evans, 1986; Makino et al., 1988; Makino 1994), rice (Makino et al., 1994) and pea (Makino 1994).

In wheat (Evans and Austin, 1986) the proportion of total leaf N allocated to Rubisco increases from approximately 15 - 23% with increasing leaf N content up to 2 g N m<sup>-2</sup>, and in spinach and rice, from 13 - 25% and 28 - 37% respectively (Makino et al., 1994). Two hypotheses have been proposed to account for the greater than proportional increase in N allocation to Rubisco with increasing leaf N content as is particularly illustrated by the response of rice (Fig. 1.1). Firstly, the resistance to the transfer of CO<sub>2</sub> from the intercellular airspaces to the sites of carboxylation within the chloroplasts may be appreciable (Evans, 1983; Evans et al., 1986). Therefore, the CO<sub>2</sub> partial pressure within the chloroplast ( $pC_i$ ) is lower for a given intercellular CO<sub>2</sub> partial pressure ( $pC_i$ ) in leaves with high photosynthetic rates, i.e. those with high N content. For example, doubling the Rubisco content from 3 to 6  $\mu\text{mol m}^{-2}$  in wheat leaves increased the *in vivo* activity by only 64% for a given  $pC_i$

(Evans, 1986). Therefore, if the balance between RuBP-regeneration and carboxylation capacities were maintained, Rubisco would have to increase by a greater relative amount with increasing N. A second hypothesis proposes that increasing the ratio of Rubisco to in particular, electron transport activity, enables the leaf to operate with a lower  $pC_i$ , resulting in a greater efficiency of water use (Farquhar and Richards, 1984; Evans, 1985). However, whilst the amount of N proportioned to Rubisco increases with leaf N content in most species (pea, rice, spinach, wheat), the absolute increases in wheat are often the smallest. An explanation for this could be that as total leaf N increases, so in parallel does the activity of carbonic anhydrase (CA; Makino et al, 1992) which could function to compensate and increase the rate of  $\text{CO}_2$  transfer. Conversely, in pea and rice, CA activity does not change in response to leaf N content (Makino et al., 1992).

The remaining enzymes of the PCR cycle are by contrast, considerably more efficient, and together, typically only account for some 6.6% of total leaf N (Table 1.1). Important to note, is the 5.4% of total leaf N invested in the thylakoid ATP-synthase, making this the next most expensive enzyme after Rubisco, in terms of N-use, and the largest single component contributing to RuBP-regeneration.

Based on this general pattern of N allocation in leaves of  $\text{C}_3$  plants grown under current  $pC_a$ , Farquhar and von Caemmerer (1982) and Field and Mooney (1986), have suggested that N should be optimally allocated to components of the photosynthetic machinery. This hypothesis can be related to the steady-state biochemical model of  $\text{C}_3$  photosynthesis (Farquhar et al., 1980; Farquhar and von Caemmerer, 1982), subsequently modified by Sharkey (1985), and discussed in the next section.

### 1.3 The steady-state biochemical model of C<sub>3</sub> photosynthesis

The aim of various mechanistic models that attempt to describe photosynthesis in C<sub>3</sub> species has been to relate studies of enzyme kinetics (principally Rubisco) and amounts of underlying biochemical components to observed photosynthetic rates. Using the *in vitro* kinetic properties of Rubisco and calculated values of  $pC_i$ , the models of Farquhar and von Caemmerer (1982), Farquhar and Sharkey (1982) and Sharkey (1985; 1989) have been used to predict how net assimilation, RuBP turnover, and electron-transport rates should vary in response to short-term (minutes to hours) changes in CO<sub>2</sub>, O<sub>2</sub>, temperature and irradiance. These models have provided good predictions of the CO<sub>2</sub>-compensation point (the value of  $pC_i$  at which carboxylation matches photorespiration), and the influence of temperature, CO<sub>2</sub> and O<sub>2</sub> on the rate of photosynthesis. They have resulted in the model that photosynthetic rate is, (1) limited in saturating light and at current or lower  $pC_a$  by the capacity of Rubisco to carboxylate RuBP, (2) limited in low light or high CO<sub>2</sub> by the capacity to regenerate RuBP (i.e. light harvesting, electron transport and PCR cycle reactions), and (3) sometimes limited at high light and saturating CO<sub>2</sub> by the capacity to regenerate PO<sub>4</sub><sup>3-</sup> within chloroplasts for ATP synthesis (Farquhar and von Caemmerer 1982; Sharkey 1985; Woodrow and Berry, 1988). Consequently, it is considered that at current  $pC_a$  and under field conditions, the control of photosynthetic rate is equally shared or co-limited by the carboxylation and RuBP regeneration capacities, as this reflects an optimal and balanced investment of resources into photosynthetic components, and therefore gives the maximal rate of photosynthesis for a given environment (Farquhar and Sharkey, 1982).

It is important to note, that the various models do contain assumptions about the relationship between the RuBP pool and the extent to which the Rubisco-binding sites will be saturated (discussed by Woodrow and Berry 1988; Sharkey 1989). The earlier models also assumed that  $pC_i$  (estimated from the transpiration rate via an analogy to Ohm's law, Farquhar and von Caemmerer 1982) provides an accurate estimate of  $pC_c$ . However, some discrepancies at high photosynthetic rates have been explained by postulating an additional resistance to CO<sub>2</sub> diffusion through the aqueous phase of cells as discussed in section 1.2 above, for which there is experimental evidence (von Caemmerer and Evans, 1991; Loreto et al., 1992).

In the current model, the net CO<sub>2</sub> assimilation rate,  $A$ , is given as:

$$A = v_c (1 - \Gamma / pC_c) - R_d \quad [1.1]$$

where  $v_c$  is the rate of carboxylation by Rubisco;  $pC_c$  is the intercellular CO<sub>2</sub> partial pressure at Rubisco active sites;  $\Gamma$  is the CO<sub>2</sub> photocompensation point; and  $R_d$  is non-photorespiratory respiration occurring in light.

The carboxylation rate,  $v_c$ , cannot be greater than; (1) the carboxylation capacity ( $W_c$ ), determined by the amount of fully activated and substrate saturated Rubisco, (2) the RuBP-regeneration capacity ( $W_j$ ), determined by the amount and activity of other PCR cycle components, and often limited by electron transport and, (3) the capacity to utilise triose-phosphate ( $W_p$ ) and regenerate PO<sub>4</sub><sup>3-</sup>, determined principally by the activity and amount of components of sucrose synthesis.

Since  $v_c$  must be less than any of  $W_c$ ,  $W_j$  and  $W_p$ ,

$$v_c = \min \{ W_c, W_j, W_p \} \quad [1.2]$$

and it is argued that the most efficient and optimal use of resources (the most limiting of which is usually N), occurs when  $v_c$  has the rate allowed by the minimum



(limiting) capacity, and all other capacities are as closely in balance as they can be, to this minimum capacity, i.e.

$$W_c \cong W_j \cong W_p \quad [1.3].$$

Consequently, if resource use is efficient (and therefore by definition the allocation of N), then biochemical function should operate to make Equation [1.3] a good description of photosynthesis. Evidence to support the model and the hypothesis that capacities determining photosynthetic rate are typically co-limiting under current growth conditions and that N is optimally allocated between components, are discussed in the following section. Evidence will be presented from (1) the short-term (minutes to hours) response of photosynthesis to altered  $pC_a$  and the modulation of the activity of components, (2) evidence obtained from studies with genetically manipulated plants, and (3) evidence from studies of long-term changes in amounts of components in response to altered irradiance and temperature.

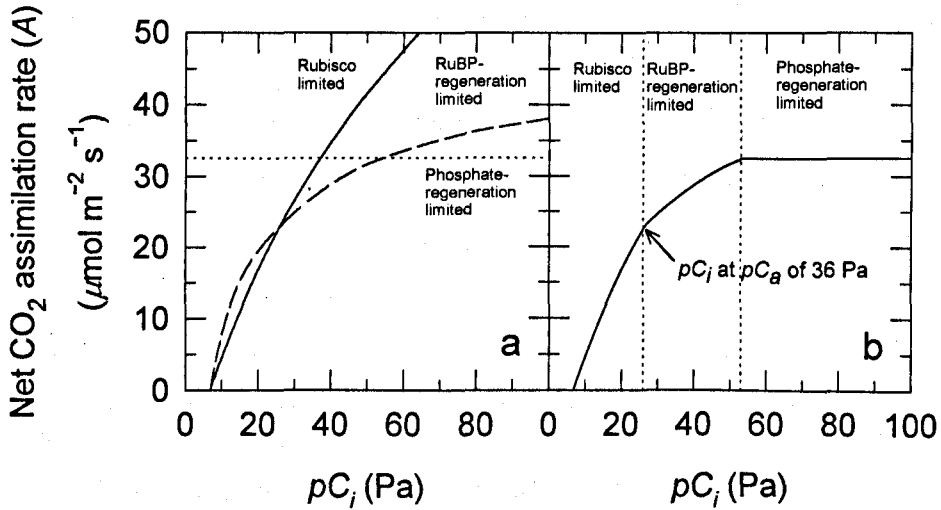
## 1.4 Evidence for the $C_3$ model and the co-limitation of capacities

### 1.4.1 *Short-term response of photosynthesis and the activity of components to elevated $pC_a$*

As outlined by the model of  $C_3$  leaf photosynthesis and illustrated in Figure 1.2a below, in intact leaves and depending on  $pC_i$ , photosynthesis is usually limited by one of three processes, (1) the capacity of Rubisco to consume and carboxylate RuBP, (2) the capacity of the thylakoid reactions to supply ATP and NADPH for RuBP-regeneration, and (3) the capacity of starch and sucrose synthesis to utilise triose-phosphates and regenerate  $PO_4^{3-}$  for photophosphorylation (Farquhar et al., 1980; Sharkey, 1985; Harley and Sharkey, 1991). The solid line of Figure 1.2b,

depicts the implication of these limitations for the response of  $A$  to short-term (minutes to hours) increase in  $pC_i$  under saturating light.

At low  $pC_i$ ,  $A$  is limited and therefore determined by the capacity of Rubisco to carboxylate RuBP, whilst the RuBP-regeneration capacity remains in excess.



**Figure 1.2** Modelled response of photosynthesis to intercellular CO<sub>2</sub> partial pressure ( $pC_i$ ). In (a), photosynthesis is modelled assuming either Rubisco capacity is limiting at all  $pC_i$  (solid line), thylakoid-dependent RuBP-regeneration capacity is limiting at all  $pC_i$  (line of long dashes), or the capacity of starch and sucrose synthesis to regenerate  $\text{PO}_4^{3-}$  is limiting at all  $pC_i$  (horizontal line of short-dashes). In (b), the corresponding  $A/pC_i$  response that would be exhibited by a leaf is depicted assuming the biochemical limitations in panel (a). Modelled after Sage (1990) using the following parameters: Rubisco  $V_{\text{cmax}} = 120 \mu\text{mol m}^{-2} \text{s}^{-1}$ ; electron transport maximum,  $J_{\text{max}} = 250 \mu\text{mol m}^{-2} \text{s}^{-1}$ , triose phosphate use rate  $= 11.2 \mu\text{mol m}^{-2} \text{s}^{-1}$ , and a light intensity of  $1800 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ . Redrawn from Sage (1994).

Since the affinities of Rubisco for CO<sub>2</sub> and O<sub>2</sub> are considered to vary little between terrestrial C<sub>3</sub> species, the main determinant of  $A$  at low  $pC_i$  will be the maximum rate of carboxylation by Rubisco ( $V_{\text{cmax}}$ ), which will be directly dependent upon the quantity of active Rubisco *in vivo* (Long and Hallgren, 1993). The initial slope of the  $A/pC_i$  response, or carboxylation efficiency ( $\epsilon$ ), therefore provides an *in vivo* measure of the activity and if fully activated, the amount of Rubisco ( $\text{mol m}^{-2} \text{s}^{-1}$ ), relating the initial slope to the capacity  $W_c$ . Subsequent studies have shown good

correlations between the initial slope or  $\varepsilon$ , and the extractable activity of Rubisco (Evans, 1983; Makino et al., 1988; Lawlor et al., 1989).

With further increase in  $pC_i$ ,  $A$  rises rapidly as carboxylation velocity is increased through greater binding of  $CO_2$  substrate at the active sites of Rubisco, until  $A$  starts to become limited by the capacity to regenerate RuBP, which becomes limiting at higher  $pC_i$ . However, a small increase in  $A$  will still occur, because as  $pC_i$  continues to increase but the partial pressure of  $O_2$  remains the same, the proportion of C lost to photorespiration declines. Under saturating light, RuBP-regeneration capacity is determined by electron transport, and because effectively all products of this process are used in RuBP-regeneration for photosynthesis and photorespiration (Habash et al., 1995), we would expect the RuBP-regeneration capacity to be determined by the amount and activity of Cytochrome *b<sub>f</sub>* complex and the thylakoid ATP-synthase, although the latter needs to be confirmed experimentally.

At saturating  $pC_i$ ,  $A$  may become limited by the capacity to regenerate  $PO_4^{3-}$ , necessary for the synthesis of ATP and subsequent production of RuBP. This limitation to photosynthesis may be particularly important in the short-term, under conditions which lead to an accumulation of soluble carbohydrates in the leaf. It is proposed that the inhibition of sucrose synthesis (or recycling of hexoses (Foyer, 1987; Goldschmidt and Huber, 1992)), leads to a large accumulation of phosphorylated intermediates which depletes the  $PO_4^{3-}$  pool, so that photosynthesis becomes limited (i.e. limited by the capacity  $W_p$  (Herold, 1980)). For example, when  $PO_4^{3-}$  is withdrawn from the medium in which isolated chloroplasts are contained, there is a decrease in the ATP/ADP ratio, glycerate-3-phosphate (PGA) accumulates, photosynthesis is inhibited (Heldt et al., 1977), and Rubisco is secondarily deactivated in order to keep the concentration of remaining Rubisco

active sites slightly below that of the RuBP pool concentration (Sage et al., 1988). Sharkey (1990) has suggested that a declining  $\text{PO}_4^{3-}$  concentration will inhibit photophosphorylation and the generation of ATP, causing the lowered ATP/ADP ratio, and decreased RuBP synthesis. Similar responses are observed when leaves are supplied with mannose to artificially sequester  $\text{PO}_4^{3-}$  as mannose-6-phosphate (Herold, 1980; Harris et al., 1983). Phosphate limitation also develops in leaves when the balance between  $\text{CO}_2$  fixation and end-product synthesis is disturbed suddenly by decreasing the  $\text{O}_2$  partial pressure (Sharkey et al., 1986; Butz and Sharkey, 1989; Sharkey, 1990), lowering the temperature (Leegood and Furbank, 1986) or adding inhibitors (Stitt and Quick, 1989).

Photosynthesis that is limited by  $W_p$  can be identified, since it will be insensitive to further increases in  $\text{CO}_2$  or decreases in the  $\text{O}_2$  partial pressure, whilst photosynthesis limited by  $W_i$  will be further stimulated by either of these treatments (Sharkey et al., 1988; Sage, 1990).

For a wide range of  $\text{C}_3$  species, analyses of leaf  $A/pC_i$  responses have frequently suggested that Rubisco activity is just sufficient to co-limit photosynthesis with the RuBP-regeneration capacity, and this is indicated by the  $pC_i$  at present  $pC_a$  being at the point of inflection of the  $A/pC_i$  response (Fig. 2.1b). One interpretation of this is that within the photosynthetic machinery, distribution of N is optimised between Rubisco and components determining RuBP-regeneration (Webber et al., 1994).

Further, in the short-term (minutes to hours), there is much evidence that the degree of activation of components can be modulated in the leaf to compensate for differential effects of environmental change on the capacities supporting photosynthetic rate (Bloom et al., 1985; Field and Mooney, 1986), thereby keeping

capacities equally co-limiting and balanced. For example, when  $W_j$  limits photosynthesis, Rubisco deactivates to the point where the RuBP concentration is maintained just above the concentration of Rubisco active sites (Perchorowicz and Jensen, 1983; Badger et al., 1984; Sage et al., 1988; Woodrow and Berry, 1988), thereby rebalancing the relationship between capacities. However, such a decrease in activity of non-limiting steps to balance that of limiting capacities, does nothing to alter the amounts of components, and therefore N-use efficiency, but rather is a mechanism for achieving homeostasis in metabolic concentrations. Nevertheless, the three capacities which determine photosynthetic rate are maintained in balance.

#### 1.4.2 *Evidence from transgenic plants*

Further evidence for the co-limitation of photosynthetic rate by the component capacities under current growth conditions and support for the unified model, can be obtained through the use of antisense RNA technology to produce transgenic plants with decreased amounts of key photosynthetic components, permitting powerful studies into the degree of control that specific components have over photosynthetic rate. Such control analysis involves asking how much a flux changes for a given change in enzyme activity, such that the flux control coefficient

$$C_E = (\delta J/J) / (\delta E/E) \quad [1.4]$$

where  $E$  is the original amount of the enzyme,  $J$  is the original pathway flux, and  $\delta J$  is the change in flux which results from a small change in the amount of the enzyme  $\delta E$ . Importantly, this approach requires that there are no major changes in the amounts of other enzymes. For an enzyme in a simple unbranched pathway,  $C_E$  can vary between zero (no control) and 1.0 (total control or limitation by that enzyme) (Kacser and Burns, 1973).

The first photosynthetic component to be manipulated by antisense technology was reported by Rodermel et al. (1988), who transformed tobacco with a full-length RNA antisense construct targeted to the small sub-unit (SSU) of Rubisco. Transformants showed a substantial decrease in *rbcS* (gene family coding for Rubisco small subunit) transcript levels as well as the amount of Rubisco protein and enzyme activity (Rodermel et al., 1988; Quick et al., 1991). Under conditions in which the plants had been grown ( $310 \mu\text{mol quanta m}^{-2} \text{ s}^{-1}$ ,  $22^{\circ}\text{C}$ ,  $35 \text{ Pa CO}_2$ ) the amount of Rubisco could be decreased to 60% of wildtype with only a marginal 6% decrease in photosynthetic rate, giving an estimated flux-control coefficient of Rubisco for photosynthesis ( $C_R$ ) of 0.1, suggesting little control. From this observation, it was suggested that the wildtype overinvested about 15% more protein in Rubisco (equating to approximately 2.6% of leaf N) than was needed to avoid a strict Rubisco limitation of photosynthesis (Quick et al., 1991). Importantly, the 40% decrease in the amount of Rubisco was offset by an increase in the activation of remaining Rubisco from 60 to 100%, as compared to wildtype.

Decreasing the amount of Rubisco further, to within 50 to 35% of wildtype resulted in strong and proportional decreases in photosynthetic rate, and below 35% of wildtype, to decreases in the amount of other components including the thylakoid ATP-synthase, NADP-malate dehydrogenase and chlorophyll, and activity of fructose-1,6-bisphosphate, with  $C_R$  approaching 1, so that Rubisco exerted almost total control. However, this was only an apparent control coefficient, given the marked decline in amounts of other components (Quick et al., 1991), and therefore is likely to be an over-estimate. Nevertheless, the observed decrease in activity and amounts of these other components, demonstrates a response by mechanisms within the plant to minimise the extreme one-sided limitation of photosynthetic

metabolism by a single enzyme (Quick et al., 1991). Thus in response, capacities were adjusted towards closer co-limitation and, with decreased amounts of components, PNUE was enhanced so supporting the model being tested.

Quick et al., (1991) concluded that the amount of Rubisco in the wildtype represents a balance between the demands of light, water and N utilisation, so that the excess allocation of N to Rubisco allows the wildtype to increase light-use efficiency by about 6%, and the instantaneous water-use efficiency by 8%, under growth conditions. However, it is reasonable that the plants used in these studies will have been genetically predisposed for growth at much higher irradiance reflective of field conditions, than the  $310 \mu\text{mol quanta m}^{-2} \text{ s}^{-1}$  employed. Under conditions of higher irradiance and 36 Pa  $\text{CO}_2$ , the photosynthetic model predicts that Rubisco, even in the wildtype, should exert considerable control. Indeed, Stitt et al., (1991), using the same plant lines, found this to be the case, obtaining a  $C_R$  value of 0.7 for wildtype plants measured at  $1000 \mu\text{mol quanta m}^{-2} \text{ s}^{-1}$  and 36 Pa  $\text{CO}_2$ . Thus, even under high light conditions, control is not exercised solely by Rubisco, and this is probably because processes such as diffusion of  $\text{CO}_2$  into the leaf then become important (Woodrow, 1994). This means that control is always shared among different processes and that the control is flexible, depending upon short-term conditions.

Sharing of control between the various capacities determining photosynthesis is further demonstrated by the studies of Price et al., (1995a and 1998), who transformed tobacco with an antisense construct directed against the transcript of the Rieske FeS protein to decrease the expression of the cytochrome *b<sub>f</sub>* complex of electron transport. Using high irradiance ( $1500 \mu\text{mol quanta m}^{-2} \text{ s}^{-1}$ ) to ensure that electron transport was the limiting component of RuBP-regeneration capacity, plants

with cytochrome *bf* complex contents ranging from 5 to 80% of wildtype were analysed. Decreases in the amount of cytochrome *bf* complex were correlated with immediate and linear decreases in photosynthetic rate at ambient  $pC_a$ , and amounts of chlorophyll, the delta subunit of the thylakoid-ATPase (ATP $\delta$ ) and Rubisco. Pool sizes of RuBP decreased in direct proportion to cytochrome *bf* complex content, whilst PGA pools were decreased to one third of wildtype content, so that there was a large decrease in the RuBP/PGA ratio, consistent with a restriction in the rate of ATP and NADPH supply. The key feature of these results was the dependence of photosynthetic rate at ambient  $pC_a$  on cytochrome *bf* complex content, so that even a small decrease in cytochrome *bf* complex content, led to an immediate fall in photosynthetic rate, confirming that the cytochrome *bf* complex may impose a rate limitation even in wildtype leaves (Price et al., 1998). Since a similar conclusion was drawn for the limitations imposed by Rubisco (Quick et al., 1991, Stitt et al., 1991), this strongly suggests that under conditions of current  $pC_a$  and high irradiance, the rate of photosynthesis is indeed co-limited by Rubisco activity (carboxylation capacity) and electron transport rate (RuBP-regeneration capacity), as suggested by the model. Again, as for the response to decreased Rubisco content, an equivalent pattern of decreases in activity and amounts of other components when the amount of cytochrome *bf* complex became severely limiting, demonstrates a response by a mechanism(s) within the plant to minimise the extreme one-sided limitation of RuBP-regeneration capacity on photosynthesis.

Prior to the work of Price et al., (1995a and 1998) RuBP-regeneration capacity had only been manipulated via antisense decreases in PCR cycle enzymes such as fructose-1,6-bisphosphatase (FBPase; Kossman et al., 1994), phosphoribulokinase (PRK; Paul et al., 1995) or glyceraldehyde-3-phosphate



dehydrogenase (GAPDH; Price et al., 1995b). A 60% decrease in FBPase activity led to a 20% decrease in the CO<sub>2</sub> saturated rate of O<sub>2</sub> evolution at 1000  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$  (Kossman et al., 1994). The activity of PRK had to be decreased by more than 65% of wildtype activity before RuBP pool sizes began to fall, and by 85% before photosynthetic rate was decreased (Paul et al., 1995). Similarly, a 60% decrease in the amount of GAPDH was necessary before the RuBP pool fell sufficiently to decrease photosynthesis (Price et al., 1995b). By contrast, the immediate decline in the RuBP pool and photosynthesis in the tobacco plants of Price et al., (1995a) as soon as cytochrome *b<sub>f</sub>* complex content was decreased below wildtype amounts, suggests that this component exerts considerable control over photosynthesis, and further, (as suggested previously by Leong and Anderson, 1984; Evans, 1987; Heber et al., 1988) that electron transport exerts more control than the enzymes of the PCR cycle in determining RuBP-regeneration capacity.

#### 1.4.3 *Evidence from long-term changes in amounts of components in response to altered irradiance and temperature*

Further evidence to support the hypothesis that N is optimally allocated between components of the photosynthetic machinery at current  $pC_a$ , can be obtained from measuring long-term changes in the amounts of components that occur as plants acclimate to irradiance (Leong and Anderson, 1984), or temperature (Makino et al., 1994).

Decreases in light intensity will decrease the thylakoid capacity to regenerate RuBP, and below the light saturation point, light harvesting capacity becomes the predominant limitation on photosynthesis (von Caemmerer and Farquhar, 1981; Evans and Seeman 1989; Sage 1990). Following long-term shading, plants often

acclimate by decreasing the content of enzymes which are non-limiting – Rubisco, Photosystem II, cytochrome *b<sub>f</sub>* complexes, and PCR enzymes – and proportionately enhance the pigment content and size of the antennae complexes in the leaf (Björkman, 1981; Anderson and Osmond, 1987; Terashima and Evans, 1988; Evans and Seeman, 1989). In low light environments, these responses increase photosynthetic rate and growth potential while substantially decreasing construction and maintenance costs of leaves relative to what would have been the case if no acclimation had occurred (Evans and Seeman, 1989; Sage, 1994), so that PNUE is enhanced.

Similarly, the photosynthetic response of leaves is strongly affected by temperature, although the dependence of each limiting capacity is not necessarily the same. In the short-term, temperature strongly influences CO<sub>2</sub> saturated photosynthesis but only slightly affects CO<sub>2</sub> limited photosynthesis (Sage and Sharkey, 1987; Labate and Leegood, 1988). This suggests that temperature affects the capacities  $W_j$  and/or  $W_p$  to a greater relative extent than the capacity  $W_c$  (Makino et al., 1994). In an experiment in which rice was grown under various long-term temperature regimes, at the lowest temperature there was evidence for a decreased investment in components determining the capacity  $W_j$ , with increased resource allocation to components determining  $W_p$  (Makino et al., 1994).

Thus in response to long-term changes in irradiance or temperature, there is evidence to suggest that an optimal distribution of resources within the chloroplast takes place, so that neither active Rubisco nor the apparatus for the regeneration of RuBP are in excess, resulting in balanced capacities and efficient N-use so that a maximal rate of photosynthesis is attained for the given environmental conditions.

## 1.5 Long-term responses to elevated $pC_a$

### 1.5.1 *Biomass and yield*

Under long-term (days to weeks) exposure at elevated  $pC_a$  many studies have shown that growth indices are enhanced and physiological parameters are affected. Kimball (1983) extensively reviewed some 430 observations of yield representing 37 mostly agricultural species, grown at  $pC_a$  ranging from 34 to 120 Pa, and concluded that on average biomass and yield was stimulated by 33% for a doubling of  $pC_a$  to 70 Pa. For wheat averaged over 14 separate experiments in which different wheat cultivars had been grown at  $pC_a$  ranging from 30 to 140 Pa, the average yield stimulation for a doubling was 51%. Most of the experiments were performed in greenhouses or growth chambers, so open fields might respond less, where nutrient availability tends to be more limiting (Lawlor and Mitchell, 1991).

### 1.5.2 *Stomatal conductance and water use*

Leaf stomatal conductance and transpiration responses are generally uniform across species to a doubling of  $pC_a$ , and inversely related to it. In a recent survey of 41 observations across 28 mainly agricultural species, stomatal conductance ( $g_s$ ) decreased on average by 20% upon long-term exposure to elevated  $pC_a$  (Drake et al., 1997), this value being similar to a 33% decrease in  $g_s$  observed in the earlier study of Cure and Acock (1986). In tree seedlings, the response of  $g_s$  is often more highly variable and often unaffected (Curtis, 1996). The mechanism of stomatal response to elevated  $pC_a$  is unclear, although it is likely to be linked through malate synthesis, since this is known to regulate anion channels in the guard cell plasma-membrane (Mott, 1990).

Decreased  $g_s$  under elevated  $\text{CO}_2$  will have a direct effect in decreasing leaf transpiration, which in the review of Cure and Acock (1986) was found to decrease on average by 23%. This was less than the 33% decrease for  $g_s$ , because as stomata close and transpiration declines, leaf temperatures increase. For example, due to decreased transpirational cooling, the canopy temperature in field grown cotton has been observed to increase by  $1^\circ\text{C}$  with growth at a  $pC_a$  of 55 Pa (Pinter et al., 1996).

### 1.5.3 *Photosynthesis*

Typically, the initial stimulation of photosynthetic rate at elevated  $\text{CO}_2$  (70 Pa) at  $25^\circ\text{C}$  ranges from 25 to 60% (Makino, 1994). However, this enhancement of photosynthetic rate at elevated  $pC_a$  in the short-term, has often been observed to decrease in the long-term (Sage, 1994 and references therein), this suppression of photosynthesis being termed acclimation, reflecting changes in the underlying capacities determining photosynthetic rate, and ultimately the amounts of components. These observations suggest that prolonged exposure to elevated  $pC_a$  leads to changes in biochemical, physiological and morphological factors which remove or offset the initial short-term stimulation. It is generally recognised that such long-term decreases in photosynthetic rate are associated with sink limitation and therefore the accumulation of carbohydrate within leaves (reviewed in Bowes, 1993; Sage 1994; Drake et al., 1997). Despite our knowledge of short-term responses to elevated  $pC_a$ , little is known of the mechanism of the long-term response (Makino, 1994). Consequently, it is convenient to divide further discussion within this section into two parts, dealing first with possible factors underlying the suppression of photosynthesis under elevated  $\text{CO}_2$ , and secondly considering aspects

of potential N optimisation in response to elevated CO<sub>2</sub>, with regard to partitioning among the photosynthetically limiting components and between sources and sinks.

#### 1.5.3.1 *Mechanisms of photosynthetic acclimation*

Two models have been proposed for the acclimation of photosynthesis at elevated  $pC_a$ , in circumstances where source production exceeds sink utilisation and carbohydrates often, but not always, accumulate within leaves.

The first model suggests that the accumulation of starch grains results in the physical disruption and damage of chloroplast membranes (Nafziger and Koller, 1976; Cave et al., 1981) thereby permanently impairing photosynthetic capacity and providing a barrier that hinders CO<sub>2</sub> diffusion to undamaged regions (Cave et al., 1981; Delucia et al., 1985). In the past this model gained support because a strong correlation between starch accumulation and suppression of photosynthetic capacity was often reported (Azcon-Bieto, 1983; Sasek et al., 1985). However, this model can no longer provide a general explanation, because Huber and Hanson (1992) found an identical inhibition of photosynthesis after warm-girdling leaves from a wild type and starchless mutant of *Nicotiana silvestris*. Further, many monocots do not accumulate significant amounts of starch in their leaves, yet still show evidence of acclimation after long-term exposure to elevated  $pC_a$ .

The second model suggests that carbohydrates can act directly by suppressing the expression of genes which encode proteins required for photosynthesis. It has been known since the early 1970s that sucrose prevents the greening of algae and cell suspension cultures (Edelman and Hanson, 1971; Dalton, 1984), and can lead to the decreased activity of Rubisco and other photosynthetic proteins (Schafer et al., 1992). In higher plants similar changes have been reported, in response to over-

expression of invertase in the apoplasm (von Schaewen et al., 1990; Stitt et al., 1991; Dickinson et al., 1991), cold-girdling (Krapp et al., 1993), glucose feeding (Krapp et al., 1991) or through growth at elevated  $pC_a$  (Besford, 1991). The regulation of gene expression has been implicated in these responses, with Sheen (1989) demonstrating that sugars strongly decrease the expression of reporter-gene constructs containing a promoter derived from photosynthesis genes, in a maize protoplast transient-expression system. In autotrophic *Chenopodium* cell-suspension cultures, Krapp et al., (1993) demonstrated that sugars lead to a rapid and reversible decrease in the steady state transcription rates of *rbcS*, *cab* (encoding the chlorophyll a binding protein) and *atpD* (encoding the delta sub-unit of the thylakoid ATP-synthase). Similarly, Krapp and Stitt (1995) reported decreased transcription for *rbcS* and *atpD* in spinach plants cold-girdled to decrease carbohydrate export from the leaf.

Under optimum environmental conditions and current  $pC_a$ , it is proposed that the rate of synthesis of photosynthates does not exceed the rate of transport and sink activity, such that there is source-sink balance. Under these conditions the expression of photosynthetic genes is mainly under the control of light and development (van Oosten and Besford, 1996). At elevated  $pC_a$  sinks begin to be saturated with C and the rate of synthesis of photosynthates progressively exceeds the rate of sucrose transport and sink strength. Consequently, sucrose accumulates in the cytosol or in the vacuoles of source leaves where it can be hydrolysed by a vacuolar invertase, resulting in an increase in the cytosolic hexose concentration, which is where the reaction products are released. Sucrose can also accumulate in the intercellular space of source leaves, where it is hydrolysed by an apoplastic invertase. Resulting hexoses are transported back to the cytosol via a hexose transporter, which appears not to be involved in the sensing of hexose in higher

plants (Krapp et al., 1993; Graham et al., 1994; Jang and Sheen, 1994; Sheen, 1994). Hexoses are then phosphorylated by a hexokinase, and it is probable, as suggested from studies in yeast, that the rate of turnover at the active site of the hexokinase might induce a signal (Rose et al., 1991), which would be transduced from the cytosol to the transcriptional machinery of nuclear photosynthetic genes via a cascade of reactions involving protein phosphorylation/dephosphorylation (Sheen, 1994). Since the major control step for gene expression in higher plants is at the transcriptional level (Kuhlemeier, 1992), fewer photosynthetic proteins would be produced and the photosynthetic capacity of the plant would be decreased. Therefore, it would seem that the regulatory metabolites responsible for the acclimation of photosynthesis to long term exposure to elevated  $pC_a$  are the hexoses, glucose and fructose. Consequently, it is hypothesised that any other factor decreasing sink-strength (low N supply, high light, low soil or air temperature) will trigger more intensively and rapidly these molecular events, and hence lead to a more pronounced photosynthetic acclimation to elevated  $CO_2$  (van Oosten and Besford, 1996).

Whilst there is evidence to support the proposed model of hexose suppression for acclimation to elevated  $pC_a$  in tomato plants grown in controlled environment cabinets (van Oosten and Besford, 1994 & 1995), there is little supporting evidence to date from wheat plants grown in the field. Nie et al., (1995b) reported only a weak correlation between increased soluble carbohydrate concentration and decreased amounts of nuclear gene transcripts in field grown wheat under FACE. Also, as no increase in glucose content *per se* was observed in leaves of wheat plants exposed to elevated  $pC_a$ , it seems more likely that as  $pC_a$  stimulates growth and demand for N elsewhere, this induces a decrease in leaf

content by some mechanism which does not involve an increase in soluble carbohydrate.

In addition to this mechanism to redress source-sink imbalance at the level of the whole plant, there has been speculation about whether there is a mechanism within leaves to redress imbalances between components of the photosynthetic machinery under conditions of elevated  $pC_a$  (Webber et al., 1994; Medlyn, 1996), as we shall discuss in the next section.

#### 1.5.3.2 *Theoretical responses for N optimisation within the photosynthetic machinery*

It has been suggested several times (Webber et al., 1994; Sage 1994; Makino 1994; Medlyn, 1996) that if optimisation of the distribution of resources between components of the photosynthetic machinery is a ubiquitous phenomenon, then adjustment of the balance between the capacities  $W_c$ ,  $W_j$  and  $W_p$ , might be expected in plants grown at elevated  $pC_a$ . Further, given the large investment of leaf N into Rubisco, strong selection pressures for adjustment of the amount of the carboxylase, given changes in  $pC_a$  in the evolutionary past, are likely.

Within the photosynthetic machinery of source leaves there is potential for increased PNUE and a re-optimisation of N, regardless of whether a decrease in photosynthetic rate has occurred. The flux of C through the PCR cycle is increased at elevated  $pC_a$  due to suppression of the oxygenase function of Rubisco and an increased saturation of active sites by  $CO_2$ , which causes the limitation to overall photosynthetic rate to shift away from the capacity  $W_c$  (determined by the activity/amount of Rubisco), to that determined by the capacity of  $W_j$  and/or  $W_p$ . If equation [1.3] is a good description of resource allocation, then given that Rubisco, a major N sink, is effectively in excess of requirements, one can envisage that in order



to restore a balance between capacities, a re-allocation of N away from Rubisco to those components most limiting photosynthetic rate could occur. This does not seem unreasonable, given the earlier examples for N re-optimisation in response to long-term changes in growth irradiance or temperature (section 1.4.3), where again, on initial exposure to these treatments, the capacities determining photosynthetic rate are initially unbalanced.

Further, because the components of RuBP-regeneration capacity account for less leaf N than Rubisco (see Table 1.1 and associated discussion), quite a small shift in N away from Rubisco to components of RuBP-regeneration will make for a substantial re-balancing (Makino, 1994). This point has been made by Woodrow (1994), discussed in terms of flux-control coefficients. However it has been disputed that flux-control coefficients add much to the analysis, since transitions between limitations by capacities is often well defined and co-limitation probably reflects heterogeneity in the leaf (Farquhar and Sharkey, 1982). A more direct approach therefore, is to simply model the amount of shift required. When this is done, for a doubling of  $pC_a$  to 70 Pa, a 30 to 40% increase in the ratio of light-saturated RuBP-regeneration capacity to carboxylation capacity has been predicted for optimal N-use efficiency (Webber et al., 1994; Medlyn, 1996).

Thus we have two levels of potential N optimisation: (1) at the whole plant level between sources and sinks, and (2) within the photosynthetic machinery of source leaves. In reality, it may be that in the earlier stages of plant development under elevated  $CO_2$ , only optimisation within the photosynthetic machinery may be necessary, developing into a co-ordinated re-distribution of N away from Rubisco and other components of the photosynthetic machinery (but still keeping this machinery optimised), only when and if, sink limitation develops in response to

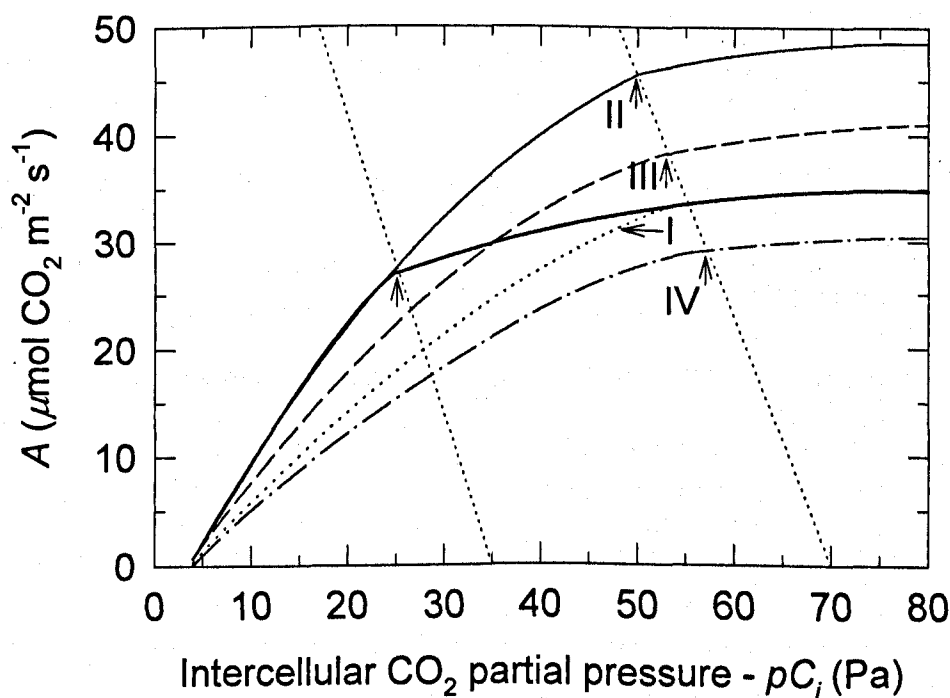
resource depletion as the plant grows and ages. The two situations can theoretically be distinguished since optimisation within the photosynthetic machinery, which would be a specific CO<sub>2</sub> effect, would manifest itself as a specific decline in the amount of Rubisco relative to other components, whilst a source-sink imbalance would be expected to result in a general decrease in all photosynthetic components.

Evidence as to whether or not optimisation within the photosynthetic machinery and/or acclimation is occurring, can be gained from the non-destructive measurement of the response of  $A$  to the intercellular CO<sub>2</sub> partial pressure ( $pC_i$ ) (Webber et al., 1994), which can be related to the model of Farquhar and co-workers described in section 1.3. As outlined previously and illustrated in Figure 1.2, at low  $pC_i$  under saturating light,  $A$  is related to the capacity  $W_c$  which produces a rapid rise in  $A$  with increase in  $pC_i$ , until photosynthesis becomes limited by the capacity  $W_j$ . As  $pC_i$  increases further,  $A$  increases slowly due to the progressive inhibition of photorespiration. Figure 1.3 below, illustrates the hypothetical patterns of  $A/pC_i$  response for the scenarios of acclimation and optimisation discussed above. It should be noted that  $A/pC_i$  measurements only give an indication of the activities, and not amounts, of various components. Although in the analysis of Farquhar and von Caemmerer (1982) it is assumed that limiting components are fully activated, this needs to be experimentally verified. Thus if  $A/pC_i$  measurements indicate that some form of acclimation/optimisation is occurring, then these have to be verified by looking for changes in the amount of components.

In the scenarios depicted in Figure 1.3, curve I (dotted line) represents the expected response if optimisation within the photosynthetic machinery occurs by means of a specific decrease in  $W_c$ . Optimisation by means of an increase in  $W_j$  is depicted by curve II (fine solid line). Curve III (dashed line) represents an

intermediate scenario, with some increase in  $W_j$  and some decrease in  $W_c$ . However, if N and sinks are strictly limiting, and there is no mechanism of optimisation within the photosynthetic machinery, then we might expect the response depicted by curve IV (dotted and dashed line), illustrating a decrease in all components.

For scenarios I, II and III the balance of capacities within the photosynthetic machinery is always restored, N is optimised and photosynthesis is utilising the additional  $\text{CO}_2$  substrate. Finally, no response at elevated  $pC_a$  may be observed, in which case the  $A/pC_i$  response will be the same as that for plants grown at 36 Pa  $\text{CO}_2$  (bold solid line in Fig. 1.3).



**Figure 1.3** The simulated response of light saturated rates of net assimilation ( $A$ ) with intercellular  $\text{CO}_2$  partial pressure ( $pC_i$ ). The solid bold line indicates the curve based on the parameters of a typical  $\text{C}_3$  leaf, developed at current  $pC_a$ . The thin solid and dashed lines illustrate the functional consequences for patterns of acclimation at 70 Pa  $\text{CO}_2$ . (I) a decrease in Rubisco activity, thus rebalancing capacities, (II) an increase in the activities (amount) of components of photosynthesis, other than Rubisco, thus rebalancing capacities, (III) a decrease in Rubisco, with re-investment into other components of photosynthesis, again rebalancing capacities, and (IV) a decrease in all components of photosynthesis, as a consequence of sink limitation. Modified from Webber et al., (1994).

## 1.6 Evidence for acclimation and N optimisation within the photosynthetic machinery in response to elevated $pC_a$

From an analysis of over 40 studies looking at the long-term effect of elevated  $CO_2$  on the short-term  $A/pC_i$  response of photosynthesis, Sage (1994), concluded that the response was clearly dependent on growth conditions. Plants grown in small pots of less than 5 L (Sage et al., 1989; Byrd and Brown, 1989; Mousseau and Saugier, 1992; Bunce, 1993; and Kriedemann and Wong, 1984) or low nutrients, normally demonstrated reduced  $A$  for a given  $pC_i$  following a doubling or even tripling of  $pC_a$  (the sink limited acclimatory response, curve IV in Figure 1.3).

Alternatively, plants grown with limiting N in large pots (Teramura et al., 1990; Ziska et al., 1991; Ziska and Teramura, 1992), or in the field (Arp and Drake, 1991; Gunderson et al., 1993), tend to show very little decrease or enhancement of  $A$  at a given  $pC_i$  to elevated  $pC_a$  (no response, Figure 1.3).

On theoretical interpretation of  $A/pC_i$  curves it was found that when nutrient limitation or pot size was not a factor, changes in the shape of  $A/pC_i$  curves were not typically observed, indicating that reallocation of resources (i.e. optimisation) had not taken place (Sage, 1994). However, a minority of studies done with potato (Sage et al., 1989), *Castanea sativa* (Mousseau and Saugier, 1992) and wheat (Hicklenton and Joliffe, 1980) did indicate a decrease in Rubisco and an enhanced capacity to metabolise photosynthates. This tended to be in studies using young plants as was the case for *Castanea sativa*, or, were grown at low irradiance as for the wheat study, or were potato plants with large storage capacities. Thus the bulk of this evidence points toward elevated  $pC_a$  mediating a response of decreased

photosynthetic rate, as a consequence of sink limitation, with no true N optimisation or rebalancing of capacities within the photosynthetic machinery.

Sage and Reid (1992) found no evidence for optimisation within the photosynthetic machinery, when French bean was grown long-term at sub-optimal  $pC_a$  (20 Pa). At sub-optimal  $pC_a$  and saturating light, carboxylation capacity would be expected to be severely limiting and RuBP-regeneration capacity in excess for photosynthesis, so that if optimisation is a ubiquitous phenomenon, a shift in N allocation away from components determining RuBP-regeneration capacity (Cytochrome *bf* complex, thylakoid ATP-synthase) towards Rubisco investment to increase the carboxylation capacity, might have been expected. Nevertheless, such experiments at sub-ambient  $pC_a$  can potentially shed light on responses to elevated  $pC_a$ , by helping to delineate direct responses to  $CO_2$ , as opposed to acclimation responses involving a general decrease in leaf N.

If truly meaningful observations in terms of a specific decrease in the amount of Rubisco relative to other components are to be observed, then Rubisco and other leaf components have to be measured relative to leaf N content. To date this has only been done in a few studies on pea and wheat (Makino, 1994) and rice (Nakano et al., 1997), where plants were grown hydroponically under controlled conditions at 36 or 70 Pa  $CO_2$ , and analysed on only one occasion at full leaf expansion. No differences were found between the two  $CO_2$  treatments for any of the relationships between amounts of Rubisco (or other components of the photosynthetic machinery) and leaf N content. These results therefore suggest that pea and rice do not have an ideal acclimation to elevated  $CO_2$  at the biochemical level (Makino, 1994). However, since measurements were only made on one growth occasion, the possibility that a response might develop later in ontogeny cannot be ruled out.

Indeed, in an experiment in which measurements were made on several occasions (Delgado et al., 1994), winter wheat grown at 70 Pa CO<sub>2</sub> had a 10% greater mean increase in photosynthetic rate over the whole experiment, compared to plants grown at 35 Pa CO<sub>2</sub>. However, there were no differences in carboxylation efficiency (indicating no change in the activity or amount of Rubisco), suggesting no re-optimisation of resources, unless changes occurred, but were too small to detect.

Nie et al. (1995a), have suggested that optimisation does occur, stating that “at elevated CO<sub>2</sub> partial pressures, subsequent to leaf emergence, the amount of Rubisco decreases as a reflection of a decreased need for N investment in this specific protein”. This conclusion is based on evidence from an experiment in which spring wheat was grown at ambient and elevated (55 Pa) CO<sub>2</sub>, under free-air CO<sub>2</sub> enrichment (FACE) in the field. For leaf 5 prior to full emergence there were no effects of growth at elevated  $pC_a$ . However, as leaf 5 became progressively shaded by emerging leaves above, there was a light acclimation response that caused a 26% decrease in the Chl a/b ratio at both partial pressures of CO<sub>2</sub>. In parallel with this, the amount of Rubisco decreased by 45% in ambient grown leaves, and 60% in elevated grown, as assessed by SDS-PAGE. Light harvesting complexes (LHC) showed no change between CO<sub>2</sub> treatments, indicating that senescence was not occurring.

For flag leaves, prior to 14 d from full emergence there was no effect of elevated CO<sub>2</sub>. However, Rubisco was subsequently seen to decrease for leaves at both CO<sub>2</sub> treatments, the decrease being greater at elevated CO<sub>2</sub>. At 36 d from emergence, Rubisco content in 55 Pa CO<sub>2</sub> grown plants was 70% less than in ambient grown plants, and at 52 d, only 20%. As before, at 36 d there was no evidence that senescence was occurring from analysis of chlorophyll content, LHC

II, or the coupling factor 1 subunit (CF<sub>1</sub>) of the thylakoid ATP-synthase, but by 52 d senescence had begun in plants under both CO<sub>2</sub> treatments. Nie et al. (1995a) argued that the decrease in Rubisco at the final stage reflected earlier senescence under elevated CO<sub>2</sub>, but that this was preceded by a specific CO<sub>2</sub> partial pressure dependent decline in Rubisco, as shown by a decrease in the ratio of Rubisco relative to other photosynthetic components, including LHC and the thylakoid ATP-synthase.

In discussion the authors propose that some of the proportional increase in loss of Rubisco at elevated CO<sub>2</sub>, is consistent with the role of Rubisco acting as a N storage protein, because at elevated CO<sub>2</sub> there are bigger (or more) grains demanding more N. Further, at elevated CO<sub>2</sub> anthesis was 2 d earlier, whilst grain filling finished 7 d earlier, probably due to slightly greater temperatures which accelerated phenological development. In addition,  $A/pC_i$  gas exchange measurements demonstrated no decrease in carboxylation efficiency at d 111, at which point 30% of Rubisco had been remobilised. This further suggests that Rubisco was in excess of requirements for photosynthesis, or that relative activities for remaining Rubisco had increased to mask losses. Nevertheless, since the apparent CO<sub>2</sub> dependent decrease in Rubisco was greater than would be expected simply as a result of senescence, this has been considered evidence for an optimal re-balancing of photosynthetic components in response to elevated  $pC_a$ .

However, in relation to the experiment of Nie et al., (1995a), Nakano et al., (1997) have shown that in young rice leaves an apparent effect of elevated  $pC_a$  appears to operate entirely via a decrease in leaf N content. Since leaves with less N contain a smaller fraction of Rubisco (see also Fig. 1.1 and associated text), there is a *de facto* readjustment in favour of RuBP-regeneration compared to carboxylation

capacity, which is dependent on N supply conditions, and would cause the ratio of Rubisco to other components to change irrespective of a direct CO<sub>2</sub> effect.

Thus the evidence to date probably demonstrates that N is not normally optimised within the photosynthetic apparatus under elevated CO<sub>2</sub>, and that when limited optimisation apparently does occur, it is often late in the life of the leaf.

## 1.7 Aims and Objectives

The principle aim of the work reported in this study was to test the hypotheses that, (1) in the event of a source-sink imbalance, growth of *Triticum aestivum* L. cv Minaret at 70 Pa CO<sub>2</sub> would allow for a decrease in N investment in leaves, thereby releasing N for the growth of roots and/or reproductive organs to redress any imbalance, and (2) more specifically and irrespective of the occurrence of (1), test whether growth at 70 Pa CO<sub>2</sub> would allow for optimal N allocation within the photosynthetic machinery, mediated via a specific decrease of 30 to 40% in the amount of Rubisco relative to other photosynthetic components. If observed, such responses would represent further improvements in N-use efficiency for the plant.

However, an additional aim, if evidence suggests that full re-balancing of N does not occur, is to determine the extent of N over-investment, as such an assessment could be used to determine the feasibility of genetically manipulating this species or others, for improved N-use efficiency in higher CO<sub>2</sub> environments of the future.

To achieve these aims, results are presented from four experiments detailed in Chapter Two, for which plants were grown at  $pC_a$  of 36, 70 or 100 Pa, and under variable N regimes to test for any interaction with CO<sub>2</sub>. Results from gas exchange



analysis are presented in Chapter Three to determine any effect of elevated  $\text{CO}_2$  on the short-term  $A/pC_i$  responses of leaves, and then direct measurement of components as a function of leaf N which determine carboxylation and RuBP-regeneration capacities respectively, are presented in Chapters Four and Five. Finally, in Chapter Six, results obtained are combined for further analysis, and to present conclusions for this study.



## CHAPTER 2

**Methodology for environmental treatments, growth of plants, routine techniques, and effect of elevated partial pressures of CO<sub>2</sub> on plant growth and yield**

### 2.1 CO<sub>2</sub> exposure facility

The CO<sub>2</sub> exposure facility used for the experiments in this study was purpose built at IACR-Rothamsted (N 51 10 48 W 0 20 30), and is contained within a glasshouse orientated north to south. The facility consists of two rows of four walk-in chambers running the length of the glasshouse, and each of 2 x 3 m in area and 2.5 m high with sealed concrete floors. Walls of 6 mm double-skinned polycarbonate sheets are held and supported within an aluminium framework, and finished with flat roofs of Perspex. Lawlor et al., (1993) found that natural radiation was decreased by approximately 30% as a result of the Perspex and glasshouse roof supporting structure. Thus, to maintain the total photosynthetically active radiation (PAR) incident on the crop over the day close to natural radiation, supplementary lighting of on average 610  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$  at crop height was provided by six 400-W SON-T lamps per chamber. These lamps were suspended from the glasshouse supporting structure but above individual chamber roofs. Following the first of the four main experiments reported in this study, natural sunlight was eliminated and nine new lamps were provided per chamber, which gave a constant PAR of 580  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$  at crop height. Lamps were programmed to switch on at daybreak and off at sunset in some experiments, or to give a fixed photoperiod, via a glasshouse control system (Envirocon 3, IACR-Rothamsted, Harpenden, UK). Glasshouse temperature was

maintained via the manipulation of air vents and heating, also controlled by an Envirocon unit. Each chamber also had its own Envirocon 3 system for controlling temperature, lights on/off, and for controlling the CO<sub>2</sub> partial pressure. Within chambers, cooling was provided by a refrigeration unit with compressor and heat exchange units outside the glasshouse. Wall mounted heaters provided background heating when required. Ambient air from outside the glasshouse was blown into chambers via individual fans and ducting, the air being filtered to remove insects. Temperature of the chamber air was sensed with platinum resistance thermometers, and temperature profiles within chambers were determined by an array of type T thermocouples. Relative humidity sensors suspended at crop height (Vaisala, Helsinki, Finland) determined humidity. Total radiation incident in each chamber was measured by 0.9 m long tube solarimeters (model TSL, Delta T Devices, Cambridge, UK), adjustable in height and always positioned at the top of the canopy. Ambient radiation was measured by a Kipp solarimeter at a meteorological site 500 m away from the facility.

The CO<sub>2</sub> content of the air within chambers was measured by individual infrared gas analysers (WMA-2, PP Systems, Hitchin, UK), which analysed samples of air taken from several points within the chamber and with a response time of approximately 45 s. Pure CO<sub>2</sub> was supplied from a bank of four 12 kg cylinders via a pressure regulator and a solenoid valve controlled by the Envirocon. Carbon dioxide was injected into the air handler inlet to ensure rapid and uniform dispersal of the gas within the chamber. The infrared gas analyser (IRGA) output signal was compared to the set point for CO<sub>2</sub> partial pressure required for individual chambers. When the IRGAs reading fell below the set point (with a dead band of 2 Pa), the solenoid was

activated via the control system and CO<sub>2</sub> would flow into the chamber until the partial pressure reached the desired target and the solenoid closed. All Envirocons were linked to a PC, where data was displayed, logged and environmental targets were set using specially developed software (ELEVCO<sub>2</sub>, G. Harrison and R. Lefevre, IACR-Rothamsted). Additional logging of thermocouple, light sensor and humidity data was performed by Campbell CR10 loggers (Campbell Scientific, Loughborough, UK).

Throughout the course of experiments described in this study, ambient and chamber IRGAs were calibrated on a weekly basis against 34, 70 and 100 Pa CO<sub>2</sub> standards (Air Products, Thetford, Norfolk, UK), and were generally never found to deviate by more than 2 Pa over a seven day period. Light sensors were found to be more stable, and therefore were calibrated on an approximate monthly basis, against a quantum sensor (LI-189; Li-Cor-Inc., Lincoln, Nebraska, USA). Of the eight chambers described, four were available for use in this study, which allowed for a maximum of two true replicates of treatments in some of the experiments. However, to further minimise bias due to small differences between chambers which might, for example affect temperature, plants were moved between chambers on a weekly basis, so that each crop stand experienced each chamber in succession; the CO<sub>2</sub> and temperature set points were re-set accordingly. This rotation of plant stands was also important in the first experiment, as plants were subjected to variability in irradiance due to natural radiation. This effect was eliminated in subsequent experiments by suspending a synthetic material screen (100%) within the glasshouse roof structure above the artificial lamps. Further, in order to minimise positional effects, plants were moved within each chamber systematically, so that each box of six pots (see 2.2 below

for further details) was at the edge of the stand for the same length of time over the course of the experiment.

## 2.2 Plant Growth

For all experiments described in this study, prior to sowing, seeds of spring wheat (*Triticum aestivum* L. cv. Minaret) were pre-germinated for 48 h in the dark at 25°C in seed trays lined with tissue paper, and kept damp with demineralised H<sub>2</sub>O. After this period, nine pre-germinated seeds were sown per 10 L, square polythene 'pot' (approximate dimensions 180 x 180 x 300 mm deep), filled with two litres of Hortag in the bottom for drainage, and 8 litres of a 1:3 sintered argillite Terra-green : Perlite mix (Silvaperl Products Ltd, Harrogate, UK). Groups of six bags were placed together in plastic boxes of dimensions 400 x 600 x 300 mm (width, length and depth respectively), which had been modified by the addition of castors, to make movements to minimise chamber effects more practical (see section 2.1 for discussion). These boxes were additionally lined in the bottom and 20mm up the inside with a continuous sheet of polythene. This was a compromise between having a barrier to prevent applied nutrients leaching straight from the bottom of bags, but not one so high up the inside of boxes that water could not escape, which otherwise may have led to anaerobic soil conditions. Boxes were arranged in arrays of 3 x 4, and each assigned to a separate chamber. Each array was therefore equivalent to a total crop area of 2.9 m<sup>2</sup> with germinated seeds sown at a density of 255 m<sup>-2</sup>. Plants were watered daily with demineralised H<sub>2</sub>O and given nutrient in the form of liquid applications (for further details see 2.3 below). In addition to rotating boxes within arrays to minimise edge effects, reflective silver screens were hung at the edges of crops at crop height to

mimic a continuous array. When bags of plants were taken for destructive measurements, other bags with plants of the same treatment were substituted, or the size of the array of boxes decreased, so that there were no gaps in the array at any time. There were four main experiments, for which details of regimes are summarised in Table 2.1. For all experiments there were two CO<sub>2</sub> partial pressures: 36 and 70 Pa in Experiments One to Three, and 36 and 100 Pa in Experiment Four; the more extreme elevation of  $pC_a$  being to ensure significant effects on Rubisco activation (see Chapter Four). In Experiment One there were three levels of N application, two levels of N application in Experiments Two and Three and one N application in Experiment Four. Principle differences between experiments were that in Experiment One, natural radiation was available resulting in more variability as demonstrated with peak values of 1400  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ , although the mean (Table 2.1) was not very different from subsequent experiments, in which natural radiation was screened out. The screening was necessary in latter experiments, because despite attempts to eliminate chamber effects by rotating plants on a weekly basis, at final harvest in Experiment One, some differences due to chamber effects were apparent for replicate treatments. Variability in radiation received by different chambers due to the direction of sunlight seemed the most probable cause of this.

A second major difference was that in Experiment One, N was applied at different rates throughout the growing season for the different N treatments, whilst for subsequent experiments, N was applied at the same rate, but stopped earlier for lower N regimes once targets were reached (see also Fig 2.1 and associated discussion). The aim of this was that a sudden and more severe N limitation might encourage a

more readily detectable re-balancing of N in elevated CO<sub>2</sub> grown plants and particularly at the biochemical level, if such mechanisms do indeed exist and function.

Experiments Two and Four, done in the winter months, were completely controlled environment experiments, and designed for gas exchange and biochemical parts of the study. Consequently, for economic reasons and because large amounts of plant material typically required for biomass studies were not needed, only two chambers were used in each of these experiments.

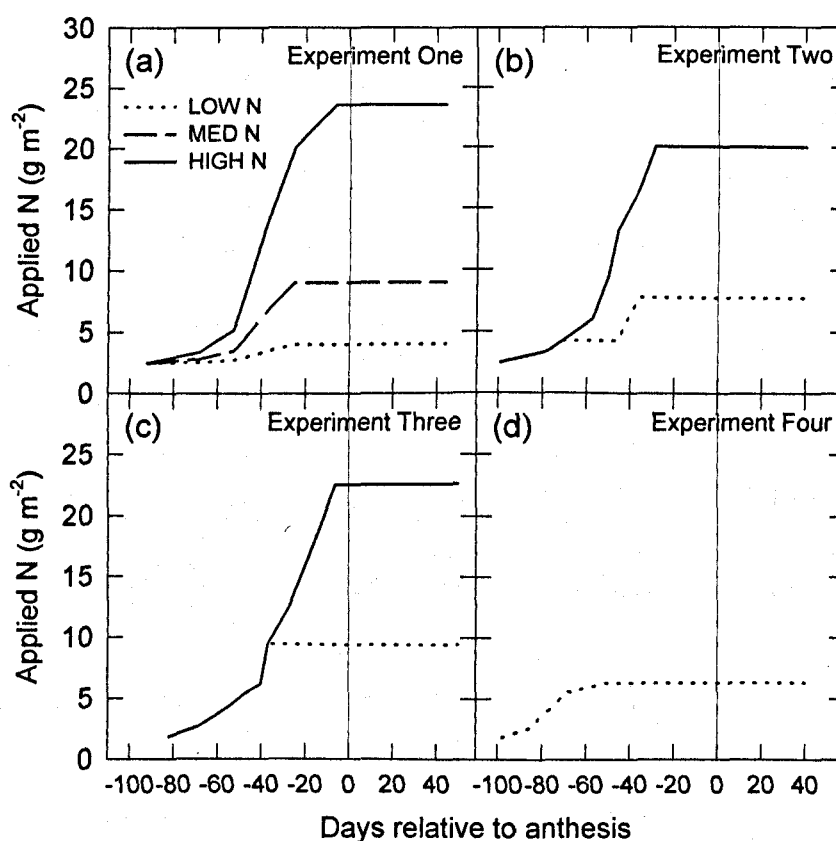


Experiment	Experimental Period	N treatments (g m <sup>-2</sup> )	CO <sub>2</sub> treatments (Pa)	Temperature regime (°C)	Radiation regime ( $\mu$ mol quanta m <sup>-2</sup> s <sup>-1</sup> )	Photoperiod regime
One	April - August 1995	4 (L) 9 (M) 24 (H)  (Applied differentially throughout experiment)	36 70	tracking outside ambient	natural radiation supplemented with artificial  average (610)	tracking natural ambient
Two	December 95 - March 96	7 (L) 20 (H)  (Applied at the same rate until 19 February)	36 70	constant day/night: 12/8 until 1 Feb, and then 20/12	artificial radiation  average (580)	constant: 12 h day until 1 Feb, and then 16 h day
Three	April - August 1996	9 (L) 22 (H)  (Applied at the same rate until 22 May)	36 70	constant day/night: 12/6 until 20 May, and then 20/7	artificial radiation  average (580)	tracking natural ambient
Four	October 96 - February 97	6 (L)	36 100	constant day/night: 16/6 until 22 November, and then 16/12	artificial radiation  average (580)	constant: 12 h day until 22 Nov, and then 14 h day

**Table 2.1.** Summary of experimental and growth regimes for the four main experiments reported in this study.

## 2.3 Nutrient applications

For all experiments, nutrients based on basal Long-Ashton solution were applied manually to pots. Target N application treatments to achieve were set at the beginning of experiments. In Experiment One, 1/5 strength nutrient solution (pH 6.3) was initially applied twice weekly between 92 and 25 d prior to anthesis, at different rates and nitrate concentrations of 1.5, 4.6 and 12.5 mM for low, medium and high N treatments respectively. Nitrate concentrations were varied by substituting chloride to provide anionic balance in the low and medium N treatments, otherwise composition was identical. After eight applications, the concentrations of all three nutrient solutions were quadrupled to loosely match nutrient supply with increased plant growth and demand. At 25 d prior to anthesis, plants had received N equivalent to 4, 9 and 20 g m<sup>-2</sup> (Table 2.1 and Figure 2.1).



**Figure 2.1** The patterns of application through time, of different N treatments for Experiments One to Four of this study. The vertical lines denote anthesis.

However, an additional application was given to the high N treatment at 7 d prior to anthesis, so that this treatment received a total of 24 g N m<sup>-2</sup>, whilst low and medium N treatments received N-free solution on this final occasion.

In subsequent experiments, N was applied at the same rate until set targets were achieved (Fig. 2.1, b - d), and thereafter N-free solution was applied until all applications ceased at approximately 10 d before anthesis. Thus for the first four weeks, 1/5 strength high N solution (12.5 mM) was applied, and subsequently quadrupled as in Experiment One. In Experiment Two (Fig. 2.1, b), the original low N treatment target of 4 g N m<sup>-2</sup> was reached very early in plant development, and subsequent plant growth was more severely inhibited than desired, for which reason an additional application was made at 35 d before anthesis. The high N target of 20 g N m<sup>-2</sup> for this second experiment was also reached approximately 25 d earlier, than in other experiments.

Regardless of specific CO<sub>2</sub> treatments, arrays of twelve boxes in each chamber, consisted of four boxes each of the low, medium and high N treatments in Experiment One, and six boxes each of low and high N treatments in Experiments Two and Three. By making all six pots of individual boxes the same N treatment, this allowed for the raising of low and medium N treated boxes on wooden blocks, so that shading effects from taller high N grown plants within the crop array, would not occur.

From Figure 2.1, it is apparent that in all experiments, there was, prior to any nutrient application, some extraneous N of on average 2.1 g m<sup>-2</sup> available to plants, and contained within the rooting medium. This had been established during an earlier provisional experiment described in section 2.8 below, and for which this extraneous

supply was taken into account when setting target applications, based on samples of rooting medium taken and tested at the beginning of experiments, using the method described in section 2.9.

## 2.4 Chlorophyll concentration

Total chlorophyll concentration within a solution of known volume was determined using 80% (v/v) aqueous acetone (Arnon, 1949). For example, to a 50  $\mu\text{L}$  aliquot of crude homogenate in a micro-centrifuge tube in darkness on ice, 95  $\mu\text{L}$  of 80% (v/v) acetone was added, the mixture immediately shaken, and centrifuged at 10000  $\times g$  for 1 minute at 4°C. The clarified solution was transferred to a 10 mm quartz cuvette, and absorbance was measured at 645 and 663 nm against an 80% (v/v) acetone blank, in a single-beam spectrophotometer (Biochrom Ultrospec II 4070, LKB, Cambridge, U.K). Total chlorophyll was calculated based on the following equations of Arnon (1949):

$$\text{Total Chlorophyll } (\mu\text{g mL}^{-1}) = 20.2 A_{645} + 8.02 A_{663}. \quad [2.1]$$

Additionally the concentrations of chlorophyll *a* and *b* were also determined:

$$\text{Chlorophyll } a \text{ } (\mu\text{g mL}^{-1}) = 12.7 A_{663} - 2.69 A_{645} \quad [2.2]$$

$$\text{Chlorophyll } b \text{ } (\mu\text{g mL}^{-1}) = 22.9 A_{645} - 4.68 A_{663}. \quad [2.3]$$

## 2.5 Soluble protein concentration

The soluble protein concentration of extracts was determined using a Coomassie Brilliant Blue G-250 Assay reagent kit (Cat No. 23200, Pierce, Rockford, Illinois, USA), following the manufacturers protocol which was based on the method of Bradford (1976).

The protein sample (typically 10  $\mu$ L) was made up to 0.5 mL with distilled H<sub>2</sub>O in a disposable 10 mm plastic cuvette. To this 0.5 mL of Coomassie reagent was added and the solutions mixed and allowed to stand for 5 min. Absorbance was read in a single-beam spectrophotometer (Biochrom Ultrospec II 4070, LKB, Cambridge, U.K) at 595 nm against reagent blanks which contained 0.5 mL of distilled H<sub>2</sub>O and 0.5 mL of Coomassie reagent. Once prepared, samples were stable for up to 1 h (manufacturers claim), but were always read within the first 30 min. The protein concentration was determined by comparison to bovine serum albumin (BSA) standards.

## 2.6 Polyacrylamide Gel Electrophoresis (PAGE)

The following stock solutions were prepared:

30% (w/v) acrylamide/0.8% (w/v) bis-acrylamide solution: 300 g acrylamide and 8 g bis-acrylamide were dissolved in distilled H<sub>2</sub>O and made up to a final volume of 1000 mL. To decolourise the solution, 3 g of activated, neutralised charcoal was added and the solution stirred for 30 min, and then filtered through two layers of Whatman No.1 paper followed by a 0.45  $\mu$ m Millipore filter. Storage was at 4°C in an amber bottle.

10% (w/v) SDS: 10 g of SDS was dissolved in distilled H<sub>2</sub>O, made up to 100 mL and stored at room temperature.

10% (w/v) Ammonium persulphate (APS): 0.1 g of ammonium persulphate was dissolved in 1 mL of distilled H<sub>2</sub>O, this being prepared on the day of use.

60% (w/v) Sucrose: 60 g of sucrose was dissolved in distilled H<sub>2</sub>O, made up to 100 mL, and stored at 4°C following filtration through a 1.2  $\mu$ m Millipore filter.

5 x Lower Reservoir Buffer (5x LRB) / Resolving gel buffer: 257 g of Tris was dissolved in 900 mL of distilled H<sub>2</sub>O, adjusted to pH 9.1 at 25°C with concentrated HCl, made up to 1000 mL and stored at room temperature.

20 x Upper Reservoir Buffer: 96.9 g of Tris, 20 g of SDS and 28 g of Boric Acid were dissolved in 900 mL of distilled H<sub>2</sub>O, adjusted to pH 8.64 at 25°C with saturated Boric acid, made up to 1000 mL and stored at room temperature.

4 x Stacking Gel Buffer: 5.24 g of Tris was dissolved in 190 mL of distilled H<sub>2</sub>O, adjusted to pH 6.1 with H<sub>2</sub>SO<sub>4</sub>, made up to 200 mL and stored at room temperature.

#### 2.6.1 *Gradient gel casting*

A 7.1 mL 10% acrylamide solution was prepared by mixing together 2.5 mL of the 30% acrylamide/0.8% bis-acrylamide stock, 1.5 mL 5x LRB, 3 mL distilled H<sub>2</sub>O, 75  $\mu$ L 10% SDS, 10  $\mu$ L TEMED and 20  $\mu$ L of 10% APS. A 7.5 mL 16% acrylamide solution was prepared by mixing together 4 mL of the 30% acrylamide/0.8% bis-acrylamide stock, 1.5 mL 5x LRB, 1.4 mL of 60% sucrose, 0.5 mL dH<sub>2</sub>O, 75  $\mu$ L 10% SDS, 5  $\mu$ L TEMED and 20  $\mu$ L of 10 % APS. Using a gradient mixer with flow of approximately 3 mL/min controlled by a peristaltic pump, these solutions were used to cast a 10-16% gradient gel between 1 mm spaced glass plates (160 x 160 mm; ATTO). Once cast, a 5 mm layer of iso-butanol was carefully added in order to straighten the top of the resolving gel. After polymerisation (approximately 30 min), the iso-butanol was washed away with distilled H<sub>2</sub>O, and a stacking gel was cast from a 7.6 mL solution consisting of 1.5 mL of the 30% acrylamide/0.8% bis-acrylamide stock, 1.88 mL of 4x stacking buffer, 4 mL distilled H<sub>2</sub>O, 75  $\mu$ L 10% SDS, 15  $\mu$ L TEMED and 150  $\mu$ L of 10% APS. A well forming comb was carefully inserted so that

the occurrence of air bubbles was minimised. After approximately 20 min, the well forming comb was removed and the gel (still between the glass plates) was wrapped in damp tissue and cling-film, and stored in a fridge overnight prior to use.

### 2.6.2 *Preparation of Samples for PAGE*

For denaturing PAGE, proteins were boiled and mixed with sodium dodecyl sulphate (SDS), an anionic detergent that confers multiple negative charges to molecules so that they become separated by an electric field. The reducing agent, dithiothreitol was used to protect protein sulphhydryl (-SH) groups, thereby removing the possibility of these moieties becoming oxidised and forming di-sulphide bridges between protein subunits.

Proteins within samples were denatured by solubilisation in 5x sample buffer which contained 250 mM Tris (pH 7.6 with HCl), 250 mM Dithiothreitol, 10% (w/v) SDS, 0.2% (w/v) Bromophenol Blue and 10% (w/v) glycerol, and which had previously been prepared and stored as 1 mL aliquots at -20°C. The concentration of either total chlorophyll or total soluble protein (see sections 2.4 or 2.5 above, respectively) was determined for samples, and on the mass basis of 20 µg of SDS: 1 µg chlorophyll or 4 µg SDS: 1 µg soluble protein, the appropriate amount of solubilisation buffer was added. Solubilised samples were boiled for 5 minutes and allowed to cool prior to loading, or alternatively frozen in liquid N<sub>2</sub> and stored at -80°C for analysis at a later date.

### 2.6.3 *Electrophoretic separation of proteins*

Discontinuous gel electrophoresis was done using the method of Laemmli (1970), so that denatured proteins were separated on the basis of molecular mass. Following the manufacturers protocol, two previously cast gels were clamped into the apparatus of an electrophoretic tank (ATTO), and the lower reservoir filled with a five-fold dilution of LRB buffer, and the upper reservoir with a twenty-fold dilution of URB buffer. Twenty micrograms of sample was added per well, as was a mixture of pre-stained molecular weight markers (Bio-Rad, Cat No. 161-0305) to others, to aid in the identification of bands and determine how fast the gel was running. A constant current of 15 mA passed through the 6% (w/v) acrylamide stacking gel, this being increased to 20 mA through the 10-16% (w/v) resolving gel for approximately 2.5 h when the dye front was typically within 5 mm from the bottom of gels.

### 2.6.4 *Coomassie staining*

Coomassie staining was used to detect protein in bands within the range of 0.1-20  $\mu\text{g}$ . Gels were removed from the electrophoretic tank and stained for 30-60 min with 10% (v/v) glacial acetic acid, 30% (v/v) methanol and 0.1% (w/v) Coomassie Brilliant Blue R-250, which had been mixed thoroughly and filtered prior to use. Gels were then destained with several washes of a solution containing 35% (v/v) glacial acetic acid, 25% (v/v) methanol and 15% (v/v) glycerol, and which had been diluted 5-fold prior to use. These steps were done in Tupperware containers placed on an orbital shaker. Destained gels were stored in water containing 1% (v/v) acetic acid.

For the quantification of bands of interest, gels were scanned (Eagle Eye, Stratagene) and images saved as TIF files. Optical densities (O.Ds) of individual bands



from these images were quantified following the manufacturers protocol of a PC software package (SigmaGel, Jandel Scientific, Sausalito, CA). Amounts of protein within samples were determined by extrapolation from calibration curves made with standards that had been loaded on the same gel and processed in the same image.

Gels were stored long-term by drying between two sheets of cellophane in a gel vacuum dryer (Bio-Rad, UK), subsequent to soaking gels overnight in a solution of 30% (v/v) methanol and 3% (v/v) glycerol to prevent either excessive swelling or cracking during the drying process.

## **2.7 Tissue N concentration**

### *2.7.1 Sample preparation*

Leaf tissue of known area (generally between 400 and 800 mm<sup>2</sup>), either harvested fresh or defrosted after storage in liquid N<sub>2</sub>, was dried in an oven for at least 48 h at 80°C, weighed and then finely ground with a pestle and mortar, transferred to a glass vial and returned to the oven for a further 24 h to drive off any moisture absorbed during the grinding process. When bulk material from whole plants was used (i.e. roots, shoots, bulk leaves, ears and grain; section 2.10.2), the tissue was first homogenised in a coffee grinder, before a smaller sub-sample was re-ground as above.

### *2.7.2 Determination by instantaneous combustion*

Using a five point balance, 5-10 mg of samples to be analysed were measured into tin weighing capsules, and using an automatic sampler, were introduced to the reactor at 1000°C, of an automatic N analyser (Carlo Erba Instruments, Milan, Italy; Model 1400). At this temperature both sample and tin capsule melt, the tin priming a

violent reaction (combustion flash) at about 1700°C, which oxidises the most thermally resistant substances. The combustion products carried by a constant flow of helium pass through an oxidation catalyst of NiO at 1000°C, and then through a 50 mm layer of silvered cobalt oxide, which removes any additional interfering products which can be produced during the combustion of halogenated substances. The mixture of CO<sub>2</sub>, N<sub>2</sub>, N<sub>x</sub>O<sub>y</sub> and H<sub>2</sub>O then passes through a reduction reactor, where nitrogen oxides are reduced to elemental N by reduced copper at 730°C. Magnesium perchlorate (anhydrous) and soda lime filters remove H<sub>2</sub>O and CO<sub>2</sub> respectively. The elemental N enters a chromatographic column and, together with the carrier gas, flows through a thermal conductivity detector which provides an electrical signal proportional to the concentration of N present, which is amplified and displayed on a digital voltmeter.

For this study, the N content of samples were calibrated against a ground Herb standard of known N content per unit mass, supplied by the Soils Department at IACR-Rothamsted, for which N content had previously been determined by several methods, including Kjeldahl digestion.

## **2.8 Preliminary experiment to determine the effects of variable N supplies on aspects of growth, photosynthesis and leaf biochemistry in spring wheat grown at 36 Pa CO<sub>2</sub>**

### **2.8.1 Introduction**

Using a broad range of N applications (0 to 24.5 g m<sup>-2</sup>) the purpose of this preliminary experiment was firstly to determine the responses and relationships of photosynthesis, some basic growth parameters and amounts of biochemical

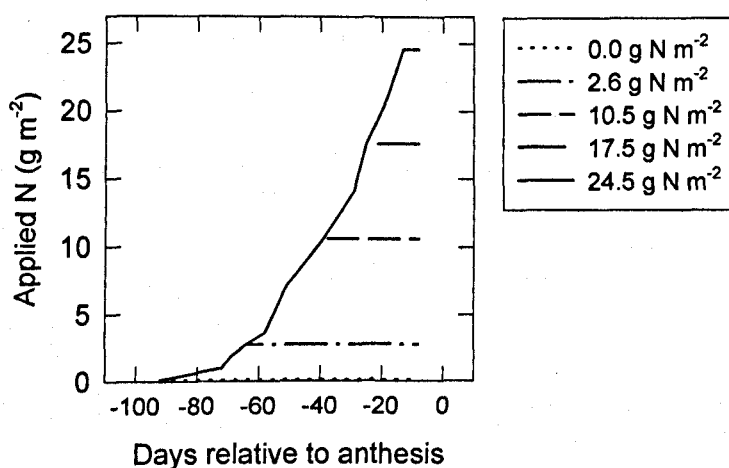
components to variable N supply that covered the anticipated range to be used in subsequent experiments. In particular it was necessary to determine whether a broad range of leaf N contents could be achieved. A second aim was to provide material for testing assays. At the time, the attainment of a broad range of leaf N contents seemed important, because if biochemical acclimation were to occur in subsequent experiments, then it was considered, not unreasonably, that effects and differences might be clearer between extremes of leaf N content and components.

### 2.8.2 *Materials and Method*

Following the protocol outlined in 2.2 above, nine pre-germinated seeds of spring wheat (*Triticum aestivum* L. cv Minaret) were sown in Terra-green:Perlite rooting medium in each of twelve 10 L plastic pots contained within two plastic boxes. Due to the small-scale nature of the experiment, the design was such that pots of different N application were contained within the same box. Therefore, to avoid cross contamination of different N applications, the sheet of polythene lining boxes in subsequent experiments (section 2.2) to prevent excess leaching and which to some degree acted as a reservoir, was omitted.

The two boxes were placed end to end in one of the chambers of the CO<sub>2</sub> exposure facility, and plants grown at  $pC_a = 36$  Pa with a 20/10°C day/night temperature regime, constant artificial radiation at plant height of 580  $\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$  and a 14 h photoperiod. Plants were well watered daily with demineralised H<sub>2</sub>O that was supplied by a purification system to which a new filter had been installed at the start of the experiment.

Five N treatments (0, 2.6, 10.5, 17.5 and 24.5 g m<sup>-2</sup>) were determined at the start of the experiment, for which two pots were used for each, except for the highest N treatment which had four. Nitrogen solution of 1/5 strength (12.5mM) based on that detailed in section 2.3 was applied at the same rate until targets were achieved, after which N-free solution was used. After the first four applications between 72 and 58 d prior to anthesis, the strength of the solution was quadrupled, and for the highest N treatments application was continued until 13 d before anthesis. These patterns of application and the total amount of N applied are summarised in Figure 2.2.



**Figure 2.2** The patterns of N application through time, as d relative to anthesis, for five treatments as denoted in the key.

Photosynthetic rate at the growth  $pC_a$  and  $A/p_i$  responses were determined in flag leaves at five d after full emergence (corresponding to 3-4 d prior to anthesis), at 20°C and saturating light, using the principles and apparatus discussed in section 3.2. Leaf samples were freeze-clamped at the growth  $pC_a$  (see 3.2 for details), and frozen and stored in liquid N<sub>2</sub> prior to the determination of leaf N content in half of the samples (section 2.7), and determination of the amount of Rubisco (4.4), and amounts of chlorophyll (section 2.4) and soluble protein (section 2.5) in the remainder.

### 2.8.3 Results and discussion

Several characteristics of the plant responses to variable N treatments for this experiment are presented in Table 2.2 below. Greater N availability enhanced tillering and resulted in increased dry weights of the plants (data not shown), reflected in greater mainstem (MS) height and increased leaf area, as indicated by values for flag leaves. Worthy of note, is the consistent uniformity of flag leaf length and area (indicated by small SEs) for plants of a given N treatment.

	Total N applied (g m <sup>-2</sup> )				
	0	2.6	10.5	17.5	24.5
Main Stem height (mm)	560 ± 7 <sup>a</sup>	740 ± 5 <sup>b</sup>	870 ± 3 <sup>c</sup>	930 ± 4 <sup>d</sup>	940 ± 5 <sup>d</sup>
Leaf length (mm)	94 ± 2 <sup>a</sup>	99 ± 3 <sup>a</sup>	191 ± 5 <sup>b</sup>	239 ± 6 <sup>c</sup>	217 ± 4 <sup>c</sup>
Leaf area (mm <sup>2</sup> )	660 ± 23 <sup>a</sup>	940 ± 46 <sup>b</sup>	2870 ± 119 <sup>c</sup>	3880 ± 138 <sup>d</sup>	3270 ± 118 <sup>d</sup>
Leaf N (g m <sup>-2</sup> )	1.1 ± 0.12 <sup>a</sup>	1.0 ± 0.08 <sup>a</sup>	1.5 ± 0.05 <sup>a</sup>	2.2 ± 0.01 <sup>b</sup>	2.4 ± 0.08 <sup>b</sup>
CO <sub>2</sub> assimilation rate (μmol m <sup>-2</sup> s <sup>-1</sup> )	14.3 ± 1.1 <sup>a</sup>	16.7 ± 0.4 <sup>a</sup>	24.5 ± 1.4 <sup>b</sup>	29.2 ± 0.9 <sup>b</sup>	31.8 ± 0.4 <sup>b</sup>
Rubisco (g m <sup>-2</sup> )	0.81 ± 0.07 <sup>a</sup>	0.97 ± 0.05 <sup>a</sup>	1.76 ± 0.11 <sup>b</sup>	2.41 ± 0.08 <sup>b</sup>	2.99 ± 0.07 <sup>c</sup>
Chl (g m <sup>-2</sup> )	0.32 ± 0.02 <sup>a</sup>	0.38 ± 0.01 <sup>a</sup>	0.52 ± 0.02 <sup>b</sup>	0.66 ± 0.02 <sup>c</sup>	0.78 ± 0.04 <sup>c</sup>

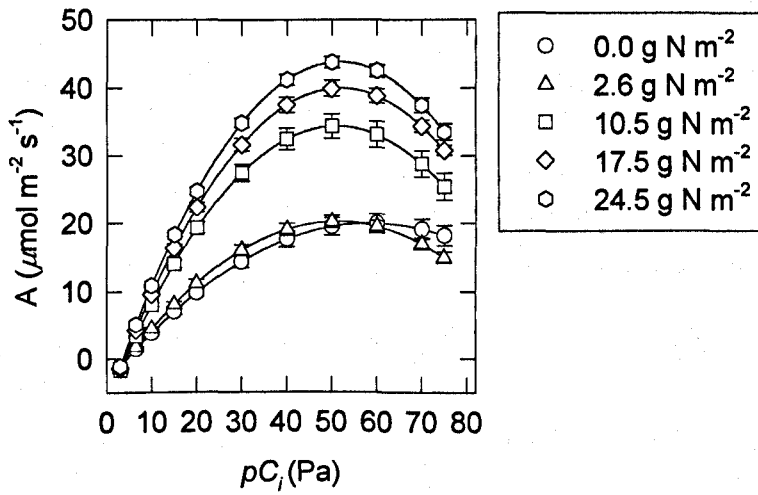
**Table 2.2** Mainstem (MS) height, leaf length and area and other characteristics for young fully expanded flag leaves prior to anthesis from plants grown at five N applications as indicated. Values are means ± SE, with n = 3 to 9. Different superscript characters indicate statistical differences between columns at  $P < 0.05$ .

Unexpectedly, plants to which no N had been applied managed to produce flag leaves and eventually reach maturity. Given that seedlings of the same cultivar grown on damp tissue paper and with only endosperm reserves to grow on reached only the second leaf stage prior to death (personal observation), suggests that extraneous N was available to all plants in this experiment, with the rooting medium being the obvious source. Consequently, equivalent samples of rooting medium were analysed for N content (procedure and results discussed in 2.9 below), and found on average to contain the equivalent of 2.1 g N m<sup>-2</sup>, demonstrating that the Terra-green rooting

medium was not inert as first thought based on implication in the manufacturers literature.

Despite this unforeseen extraneous supply of N, at least a 12-fold difference in N applications for this experiment was nevertheless achieved. However, despite this large difference in N applications, there was only slightly more than a 2-fold difference in flag leaf N content across treatments (Table 2.2), in turn determining that there were no more than 3.5-fold differences in CO<sub>2</sub> assimilation rate, Rubisco and chlorophyll contents. This same finding has previously been observed by Evans (1983) for wheat, for which he reported that such a response was in marked contrast to species such as *G. hirsutum* (Wong, 1979) and *Phaseolus vulgaris* (von Caemmerer and Farquhar, 1981), in which variable N supply resulted in plants with widely varying assimilation rates and N contents per unit leaf area. In large part, the explanation for this relative insensitivity of wheat leaf N content, is that the main effect of a high N supply is on increased tillering (as observed in this experiment), and depending upon the continuation of N supply, by the number of tillers which go on to survive to maturity. Possibly, broader ranges of leaf N contents might be attainable by using unicum varieties.

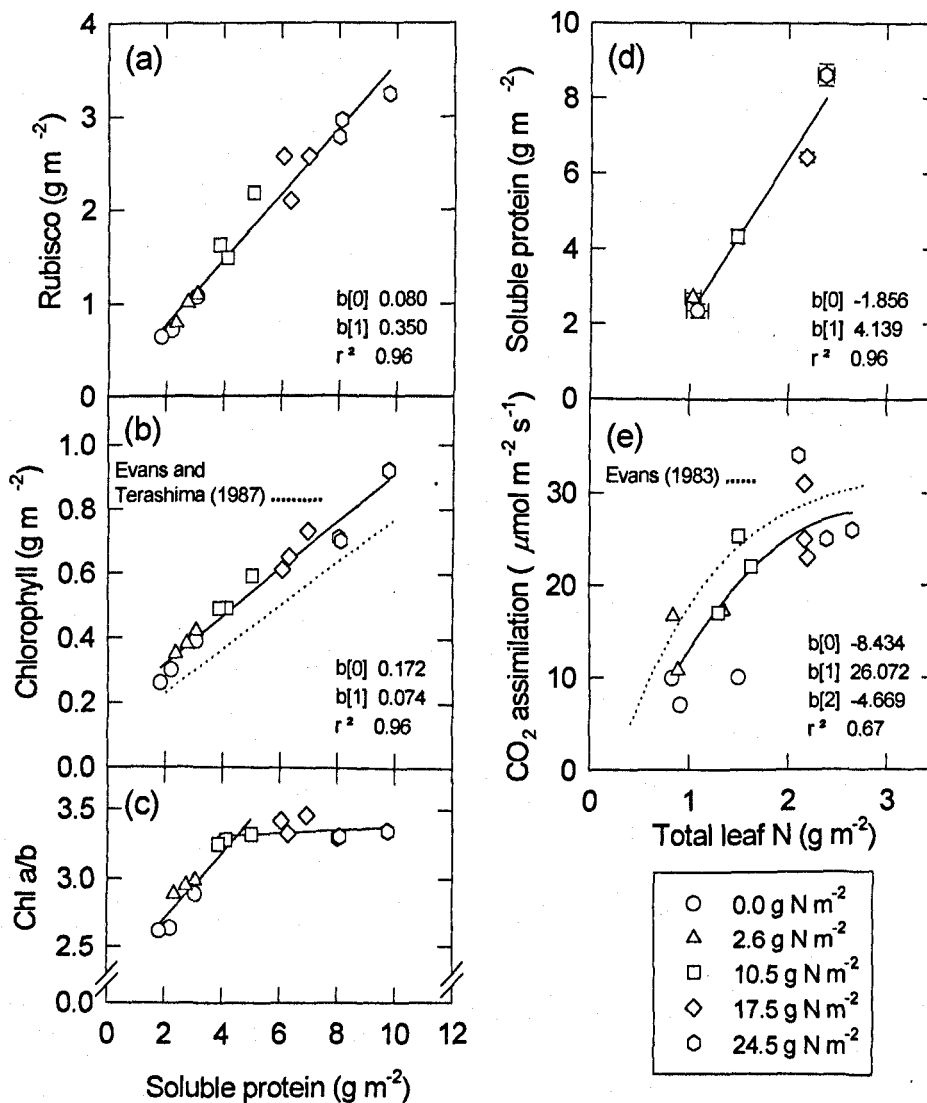
The mean  $A/pC_i$  response for four replicate flag leaves of each N treatment measured shortly (2-3 d) after full expansion but 3-4 d prior to anthesis are shown in Figure 2.3. Despite only modest increases in leaf N content from N applications during growth, applications nevertheless did result in increased carboxylation efficiency (denoted by the linear slope at low  $pC_i$ ) and  $A_{max}$ , and therefore overall photosynthetic capacity.



**Figure 2.3** Assimilation rate versus intercellular  $\text{CO}_2$  pressure ( $pC_i$ ) for flag leaves of wheat grown at a range of N applications as denoted by the key, and measured between full expansion and anthesis. Bars are 1 x SE, with  $n = 4$ .

This same pattern of  $A/pC_i$  response as a function of N applied during growth has been reported for flag leaves of winter wheat, also measured between full leaf expansion and anthesis (cv. Avalon; Lawlor et al., 1989). In contrast to these two independent observations, Evans (1983) previously reported that the  $A/pC_i$  responses of spring wheat grown with either abundant or no nitrate supply, were essentially identical from full emergence until at least one week after anthesis.

However, in agreement with Evans (1983) and Lawlor et al., (1989), for the  $A/pC_i$  responses reported here, there was no significant change with increasing leaf N content for the ratio of  $A_{\text{max}}$  to carboxylation efficiency (data not shown), suggesting that the allocation of N into components determining carboxylation and RuBP-regeneration capacities remained proportional, at least so far as can be determined from these  $A/pC_i$  responses.



**Figure 2.4** Amounts of Rubisco, Chl and Chl *a/b* ratio versus soluble protein content, and amounts of soluble protein and the rate of CO<sub>2</sub> assimilation at saturating light and  $pC_a = 36$  Pa versus leaf N content. Measurements were determined in flag leaves between full emergence and anthesis, and on plants grown at five different N applications as indicated by the key. For the relationship between soluble protein and leaf N content (panel d), points are the mean of three soluble protein values versus the mean of three N values, with 1 x Std Err shown for both axes of each. Dashed lines represent responses for the same relationship obtained by Evans and Terashima (1987) and Evans (1983), for panels b and d respectively, and are shown for comparison.

The proportions of Rubisco, chlorophyll and the Chl *a/b* ratio to soluble protein content measured in flag leaves were all independent of N treatment (Fig. 2.4 a to c), with the relationships for Rubisco and chlorophyll being approximately linear, and the relationship of the latter to soluble protein content being comparable



to that obtained in spinach (Evans and Terashima, 1987). The Rubisco to soluble protein relationship was such, that Rubisco represented 35% of the former.

The relationship between the Chl  $a/b$  ratio and soluble protein content was biphasic, the ratio increasing from 2.6 to 3.4 with an increase in soluble protein content from 2 to 4 g m<sup>-2</sup>, the ratio thereafter remaining largely independent of any further increases in soluble protein content. Since the Chl  $a/b$  ratio reflects the relative abundance of light harvesting chlorophyll proteins to reaction centre complexes (Evans and Terashima, 1987), this implies that at a low soluble protein content and low leaf N content proportionately more N is invested into light harvesting complexes than reaction centres. This effect is well known, and reflects the fact that even leaves with low N content absorb nearly all incident light and have a relatively larger light harvesting content, but leaves with more N and higher  $A_{\max}$  have more photosystems per unit chlorophyll.

As Lawlor et al., (1989) observed, there was a strong correlation and linear relationship between soluble protein and total leaf N content, with soluble protein increasing with increasing leaf N. However, since the relationship intercepts the x-axis at 0.5 g N m<sup>-2</sup>, this implies that with increasing leaf N content, proportionately more N is invested into soluble proteins than insoluble, as has been previously reported (Evans, 1989).

Despite continuous increases in Rubisco and Chl content with increases in soluble protein and by definition N content, the rate of CO<sub>2</sub> assimilation (Fig. 2.4, e) did not increase much with increases in leaf N content above approximately 2 g N m<sup>-2</sup>, with both this value and the pattern of response, for which a second order polynomial regression gave the best fit, being very similar to observations made

previously by Evans (1983), also for wheat. Consequently, a single relationship for this response appears to be justified, because regardless of whether a change in leaf N content was brought about by different nutrition, the effect on the relationship was the same. Evans (1983) further demonstrated that this relationship in wheat was also independent of seasonal conditions and leaf senescence.

Thus, from these results an understanding and comparison with previous data in the literature had been made for the various relationships investigated, and the expectation of leaves with widely varying leaf N contents eliminated as a possibility.

## **2.9 Determination of extraneous nitrate-N and ammonium-N content in Terra-green/Perlite rooting medium**

### **2.9.1 *Introduction***

Potassium chloride solution extracts all nitrate and ammonia from soils without decomposing any organic N compounds that might be contained within it. The extracts obtained can be analysed for nitrate-N (after reduction to nitrite-N) and ammonia by colourimetry using an Alpkem rapid flow analyser.

### **2.9.2 *Materials and method***

From batches of bags of Terra-green used for respective experiments and selected randomly, 50 g samples were placed into wide necked polythene bottles, to which 125 mL of 2M KCl (AR grade, low in  $\text{NO}_3\text{-N}$  and  $\text{NH}_4\text{-N}$ ) was added, and bottles sealed and shaken for 2 h at approximately 120 strokes per minute on a reciprocating shaker. Bottles were removed and allowed to stand for 30 min, and then

the supernatant was decanted through Whatman No.1 filter paper and collected, after the first 10 mL of filtrate had been discarded. Filtrates were then sent to colleagues within the Soils Department at IACR-Rothamsted, who determined nitrate-N and ammonium-N concentration using an Alpkem rapid flow analyser. In addition to Terra-green, samples of Perlite and Hortag were also sent for analysis.

### 2.9.3 Results and discussion

From the results presented in Table 2.3 below, it is clear that Perlite was completely inert, as was essentially Hortag, with only traces of  $\text{NO}_3\text{-N}$  and  $\text{NH}_4\text{-N}$  contained within it, and further, with only traces of  $\text{NO}_3\text{-N}$  within Terra-green.

Experiment	Sample type	Sample No.	$\text{NO}_3\text{-N}$ ( $\text{g m}^{-2}$ )	$\text{NH}_4\text{-N}$ ( $\text{g m}^{-2}$ )	Total N ( $\text{g m}^{-2}$ )
One	Perlite	1	0.0	0.0	0.0
	Hortag	1	0.012	0.0014	0.0134
	Terra-green	1	0.028	2.48	2.51
		2	0.023	2.48	2.50
		3	0.034	2.59	2.63
		4	0.017	2.58	2.60
		5	0.006	2.47	2.48
Two	Terra-green	6	0.011	2.47	2.48
		1	0.12	1.50	1.62
Three	Hortag	1	0.25	0.11	0.14
	Terra-green	1	0.030	1.80	1.83
		2	0.018	1.61	1.63
		3	0.040	1.75	1.78
		4	0.018	1.92	1.93
		5	0.012	1.81	1.82

**Table 2.3** Summary for amounts of nitrate and ammonium nitrogen extracted in KCl from samples of Perlite, Hortag and Terra-green used as the rooting medium to grow plants in experiments of this study. Results are expressed on a  $\text{g N m}^{-2}$  basis, taking into account the specific rooting medium composition and pot volumes used in this study.

However, Terra-green contained a substantial amount of  $\text{NH}_4\text{-N}$ , which taken as a mean from the three experiments analysed, represented an additional  $2.1 \text{ g N m}^{-2}$

(derived from 39mg N per litre Terra-green) for the specific rooting medium mixture used in this study.

The available N in Terra-green also explains the small discrepancy between N uptake and N applied in Mitchell et al., (1993) where zero content was assumed. Clearly in Experiment One of this study, where the lowest target for N to apply was 4 g m<sup>-2</sup>, a lack of awareness of the extraneous N in Terra-green would have resulted in a N availability to plants 55% again above that which had been applied (assuming the average of 2.1 g N m<sup>-2</sup>).

## **2.10 The effects of elevated partial pressures of CO<sub>2</sub> on plant growth and yield**

### **2.10.1 *Introduction***

One of the aims of Experiments One and Three of this study, was to test the hypothesis of whether growth at elevated CO<sub>2</sub> would allow for a decreased N investment in leaves, thereby releasing N for further growth in roots and/or reproductive structures. An additional aim was to test the hypothesis that more response might be expected in plants grown with the greatest N limitation.

### **2.10.2 *Materials and Method***

The growth facility, and method of plant cultivation have already been discussed (sections 2.1 and 2.2 respectively), as has the method and rates of N application (section 2.3, Fig. 2.1). The details of regimes used for these two experiments are outlined in Table 2.1, principle differences being that in Experiment

One natural radiation was available and N treatments were applied at different rates throughout the growing season, whilst in Experiment Three, only artificial radiation was present, and N was applied at the same rate but stopped earlier for the low N treatment.

For crop measurements in Experiment One, the number of stems (mainstem and tillers combined) and mainstem leaf number in six pots for each treatment were counted every week up to anthesis. For phenological measurements, emergence was assessed visually, as was the percentage anthesis on a daily basis through the flowering period, by randomly selecting and scoring 20 ears per treatment. Water-soluble carbohydrate content (glucose, fructose, sucrose and fructans) was determined in leaves and stems at anthesis using an enzymic method based on Jones et al., (1977).

In Experiment One, eight pots per treatment were destructively harvested at terminal spikelet, anthesis and physiological maturity, and for Experiment Three, at anthesis and physiological maturity only. On each occasion, the number of stems, their dry mass and the dry mass of the roots, green leaves and collectively non-green leaves and dead material were determined. Additionally, for later harvests, the number of ears, ear dry mass, number of grains and grain dry mass were measured. Tissue N concentrations were then determined for these samples (section 2.7): all three harvests in Experiment One, and final harvest in Experiment Three. Data were analysed for treatment effects using  $\text{CO}_2 \times \text{N}$  ANOVA (Microsoft Excel).

### 2.10.3 Results and discussion

As found previously for winter wheat in the Rothamsted CO<sub>2</sub> exposure facility (Mitchell et al., 1993), for Experiments One and Three of this study in which spring wheat was grown, CO<sub>2</sub> enrichment either did not affect phenological development, or gave small inconsistent effects (Table 2.4). Van Oijen et al., (1998) similarly concluded that phenological development was not affected by growth at elevated CO<sub>2</sub>, in two field like, cooled open-top-chamber (OTC) experiments, in which the same spring wheat cultivar Minaret was used.

N treatment		Low		Medium		High	
CO <sub>2</sub> (Pa)	Expt	36	70	36	70	36	70
Emergence	1	7 April	7 April	7 April	7 April	7 April	7 April
	3	15 April	15 April			15 April	15 April
Terminal spikelet	1	19 May	19 May	19 May	19 May	19 May	19 May
	3	1 June	1 June			1 June	1 June
50% anthesis	1	25 June	27 June	27 June	27 June	29 June	29 June
	3	30 June	1 July			30 June	1 July
100% anthesis	1	27 June	29 June	29 June	29 June	30 June	30 June
	3	2 July	3 July			2 July	3 July
Start grain fill	1	7 July	7 July	7 July	7 July	10 July	10 July
	3	12 July	12 July			12 July	15 July
End grain fill	1	24 July	3 Aug	3 Aug	31 July	31 July	3 Aug
	3	8 Aug	8 Aug			8 Aug	8 Aug

**Table 2.4** Phenology dates for Experiments One and Three. Crops were pre-germinated and sown on 31 March 1995 and 9 April 1996 respectively.

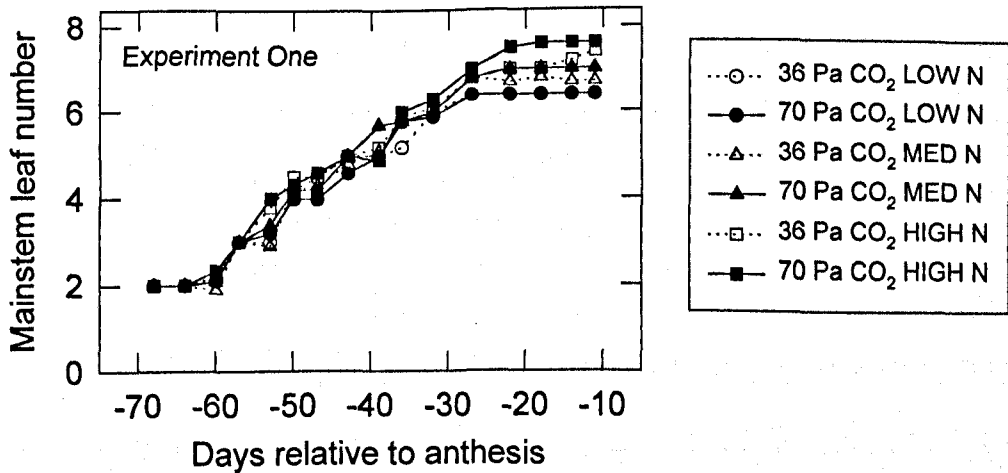
Low N supply did bring forward the date of anthesis in Experiment One, in which plants had deficient N throughout growth, but not for the low N grown plants of Experiment Three where the N limitation only occurred shortly before terminal spikelet. The greatest difference in the duration of growth phases between the two experiments was for the terminal spikelet to 50% anthesis phase, which was 11 d shorter in Experiment Three, compared with Experiment One. The relative

difference between experiments was decreased but not eliminated, by expressing on a thermal time basis with base temperature 0°C (Table 2.5).

Growth stage	Experiment One		Experiment Two	
	Days	Thermal time	Days	Thermal time
emergence - terminal spikelet	42	437	46	570
terminal spikelet - 50% anthesis	41	558	30	490
50% anthesis - start grain fill	9	156	12	197
start grain fill - end grain fill	25	513	26	538

**Table 2.5** Phenological duration's in days and thermal time (base temperature of 0°C) for high N treatments, averaged over CO<sub>2</sub> treatments for experiments.

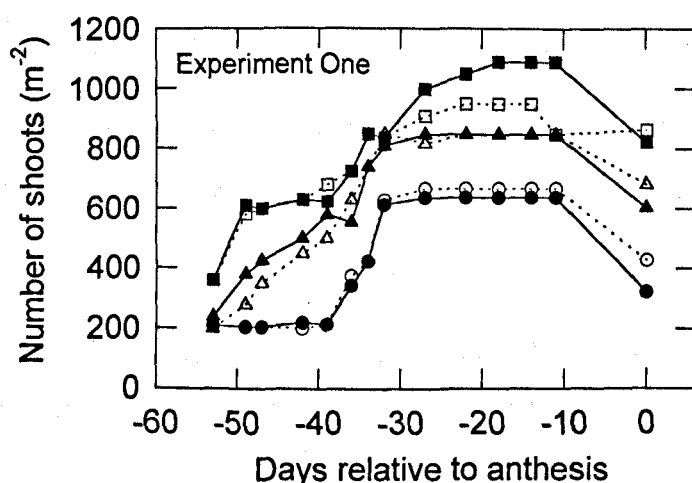
In the period up to anthesis, mainstem leaf number was not affected by growth at elevated CO<sub>2</sub>, but the final leaf number was lower for the low N treatment (4 g m<sup>-2</sup>) in Experiment One (Fig. 2.5).



**Figure 2.5** Mainstem leaf number through development prior to anthesis for Experiment One.

Final leaf number was not significantly affected by CO<sub>2</sub> or N treatment in Experiment Three (data not shown).

In Experiment One, the number of shoots (mainstem and tillers combined) was greatly affected by N treatment, but the only persistent CO<sub>2</sub> effect was to increase the survival of shoots in the period up to 10 d before anthesis at high N (Fig. 2.6).



**Figure 2.6** Number of shoots (mainstem and tillers combined) through development for Experiment One. Symbols and lines as in Fig. 2.5.

However by anthesis, growth at 70 Pa CO<sub>2</sub> had significantly decreased shoot numbers ( $P < 0.05$ ) by 25, 11 and 5% respectively in going from low to high N treatments (Fig. 2.6), reflecting greater tiller mortality and presumably remobilisation of the resources that they contained. This is based on the observation that for mainstems and tillers that remained, a significantly greater percentage ( $P < 0.001$ ) carried ears on 70 Pa CO<sub>2</sub> grown plants (Table 2.6). A similar observation was made by Mulholland et al., (1997), again growing the cultivar Minaret in OTC's: no beneficial effect of elevated CO<sub>2</sub> was observed on either the number of tillers produced, or the number surviving to maturity, but similar to this study, there was a small (although in their case non-significant) increase in the number of ear-bearing tillers under elevated CO<sub>2</sub>. Likewise, van Oijen et al., (1998)



observed no significant effect of elevated CO<sub>2</sub> on tiller density in two consecutive experiments with Minaret. These observations contrast with those of Sionit et al., (1981), Hocking and Meyer (1991) and Mitchell et al., (1993), who observed a CO<sub>2</sub> stimulation in the number of shoots surviving through to maturity. However, in the first two studies, ample nutrient solution was applied daily up to anthesis so that tiller mortality might be expected to have been negligible, and in the latter study winter wheat was grown, so that a longer growing season was available to allow a greater abundance of assimilate to accumulate at elevated CO<sub>2</sub>, thereby permitting more tillers to survive (Lawlor and Mitchell, 1991).

N treatment CO <sub>2</sub> (Pa)	Low		Medium		High		F probability		
	36	70	36	70	36	70	N	CO <sub>2</sub>	NxCO <sub>2</sub>
% stems with ears	56	80	67	76	60	78	NS	<0.001	NS

**Table 2.6** Percentage of stems (mainstem and tillers combined) carrying ears at anthesis in Experiment One.

Canopy leaf area index (LAI) at anthesis was not significantly affected by CO<sub>2</sub> in either experiment ( $P>0.05$ ), but was dramatically affected by N application in both (Table 2.7). Mulholland et al., (1997) similarly observed no significant effect of CO<sub>2</sub> on canopy LAI, although the LAI of the mainstem was greater throughout the growing season under elevated CO<sub>2</sub>.

N treatment CO <sub>2</sub> (Pa)		Low		Medium		High	
		36	70	36	70	36	70
LAI	1	0.45	0.53	1.88	1.75	4.53	5.17
	3	2.38	2.11			4.27	4.14

**Table 2.7** Green Lamina Area Index at anthesis for experiments One and Three.

Total crop dry mass including roots (Fig. 2.7), was stimulated progressively more through the season by elevated CO<sub>2</sub> in both experiments. In Experiment One there was no significant CO<sub>2</sub> effect at terminal spikelet (d -36), but by anthesis the effect was significant ( $P<0.05$ ) in both experiments.

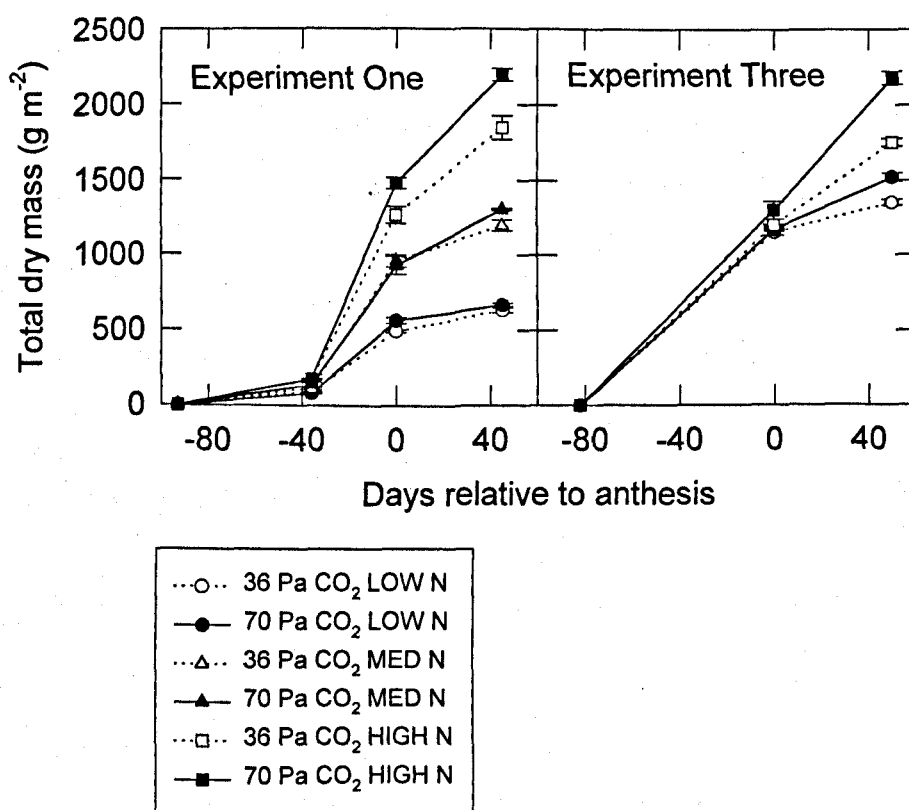
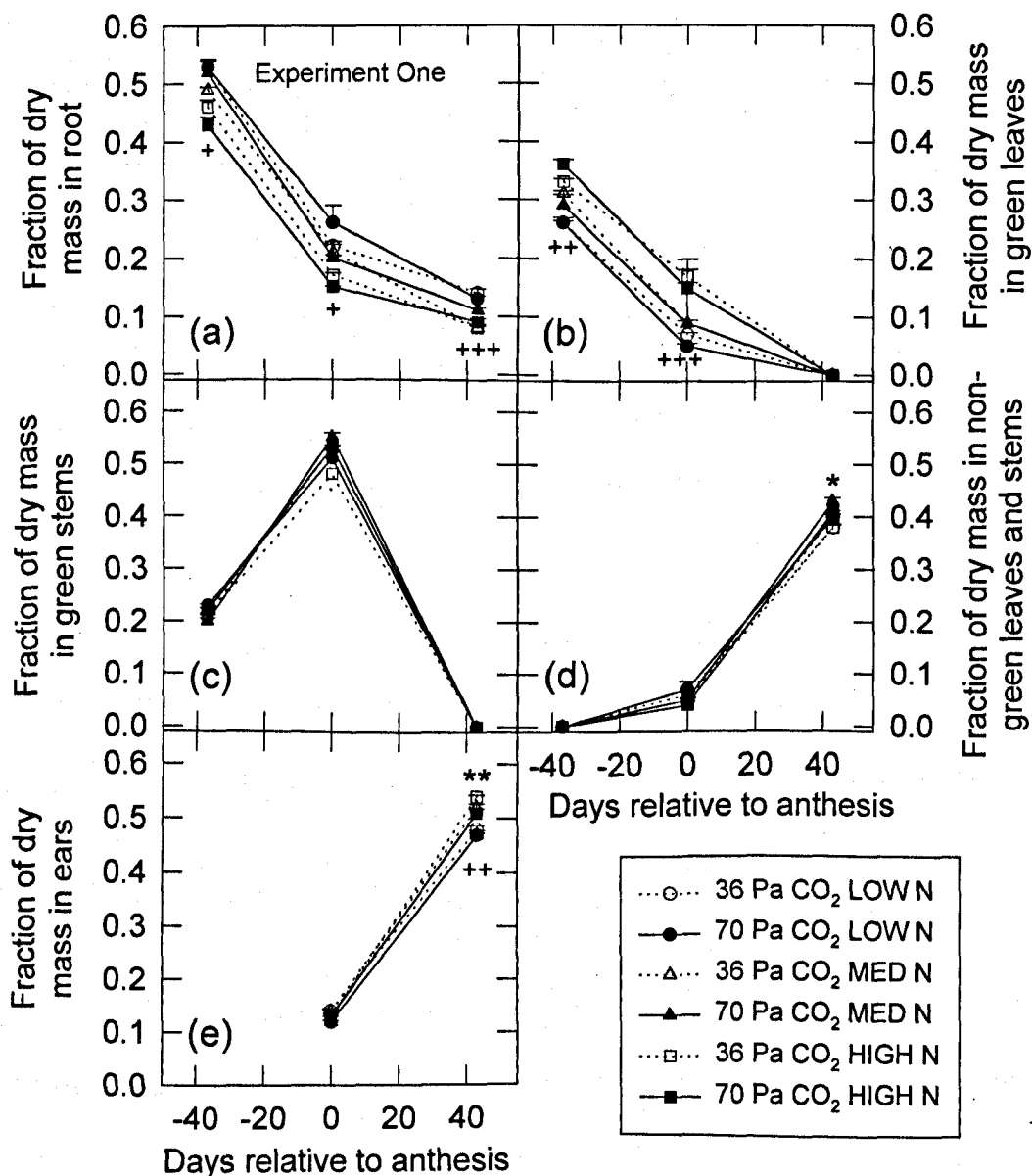


Figure 2.7 Total crop dry mass, including roots, through time. Error bars represent  $\pm$  SE of 6-8 replicates.

In both experiments there was a clear positive interaction between CO<sub>2</sub> and N supply in both relative and absolute terms for final biomass (significant at  $P<0.05$  and  $P<0.005$  for experiments One and Three respectively). The CO<sub>2</sub> stimulation of biomass for Experiment One was +6% (95% confidence intervals, CI: -1%, +14%), +9% (CI: 0%, +18%) and +18% (CI: +4%, +34%) respectively for the 4, 9, and 24 g N m<sup>-2</sup> treatments. For Experiment Three, the values were +10% (CI: +5%, +15%) and +21% (CI: +13%, +38%) for 8 and 21 g N m<sup>-2</sup> treatments respectively. The smaller confidence intervals were due to smaller variability between replicates in

Experiment Three, in which natural radiation was screened out. In general, the effect of CO<sub>2</sub> was very similar for equivalent N treatments in Experiment One and Three (Fig. 2.7), given that the 9 g N m<sup>-2</sup> medium treatment of Experiment One is similar to the 8 g N m<sup>-2</sup> low treatment of Experiment Three (Table 2.1). In support of these findings, Mulholland et al., (1997) observed a total biomass stimulation of 23% for Minaret grown at 68 Pa CO<sub>2</sub> and ample nutrition, and Havelka et al., (1984), a 20% increase in biomass for wheat grown at 120 Pa CO<sub>2</sub>.

In Experiment One, the fraction of crop dry mass in the root for all three harvests was always significantly greater ( $P < 0.05 - 0.001$ ) at low N (Fig. 2.8 a, below), at the expense of a significantly smaller ( $P < 0.01$ ) fraction of crop dry mass to leaves at terminal spikelet and anthesis (Fig. 2.8 b), and a significantly smaller ( $P < 0.01$ ) fraction of crop dry mass in the ear at final harvest (Fig. 2.8 e). However, neither N or CO<sub>2</sub> treatments had any significant effect on the fraction of crop dry mass distributed to any other plant part at terminal spikelet or anthesis. At final harvest for Experiment One, CO<sub>2</sub> did exert a significant effect ( $P < 0.01$ ) on the fraction of crop dry mass distributed to the ear, being surprisingly greater for plants grown at 36 Pa CO<sub>2</sub>. Upon breakdown of the ear into components of grain and chaff, the effect was found to be solely attributable to a greater fraction of crop dry mass in the chaff (Table 2.7). This observation most likely reflects a slightly later start date to, or slower rate of grain filling in 36 Pa CO<sub>2</sub> grown plants, since whilst not significant, the fraction of dry mass in grain at final harvest for 36 Pa CO<sub>2</sub> grown plants was always lower (Table 2.7).



**Figure 2.8** The fraction of total crop dry mass distributed to different plant parts, for the three harvests of Experiment One. Each point represents the mean of eight samples, and where visible error bars = 1 x Std Err. Asterisks denote significant effects of CO<sub>2</sub> for the occasion indicated: \* = ( $P < 0.05$ ), \*\* = ( $P < 0.01$ ) and \*\*\* = ( $P < 0.001$ ). Addition signs denote significant effects of N for the occasion indicated: + = ( $P < 0.05$ ), ++ = ( $P < 0.01$ ) and +++ = ( $P < 0.001$ ).

In contrast to Experiment One, the fraction of crop dry mass in roots at anthesis was not significantly affected by either N or CO<sub>2</sub> treatment in Experiment Three (Table 2.8), reflecting the similar supply of N to this stage of development, for both the low and high N treatments (Fig. 2.1, a & c).

**Table 2.7** Fraction of total crop dry mass distributed to the ear and its components at final harvest in Experiment One. Values are means of four replicate pots, each pot containing nine plants. Values in parentheses are 1 x SE.

CO <sub>2</sub>	Low N		Medium N		High N		F Probability		
	36	70	36	70	36	70	N	CO <sub>2</sub>	NxCO <sub>2</sub>
Ear	0.48 (±0.014)	0.47 (±0.008)	0.52 (±0.014)	0.47 (±0.018)	0.54 (±0.005)	0.51 (±0.015)	<0.01	<0.01	NS
<i>of which:</i>									
Grain	0.33 (±0.010)	0.38 (±0.008)	0.33 (±0.022)	0.34 (±0.019)	0.36 (±0.007)	0.38 (±0.015)	NS	NS	NS
Chaff	0.15 (±0.006)	0.09 (±0.003)	0.19 (±0.013)	0.13 (±0.006)	0.18 (±0.003)	0.13 (±0.004)	<0.01	<0.001	NS

**Table 2.8** Fraction of crop dry mass distributed to different plant parts, for the two harvests of Experiment Three. Values are means of four replicate pots, each pot containing nine plants. Values in parentheses are 1 x SE.

*Anthesis:*

CO <sub>2</sub>	Low N		High N		F Probability		
	36	70	36	70	N	CO <sub>2</sub>	N x CO <sub>2</sub>
Leaves	0.09 (0.003)	0.08 (0.007)	0.14 (0.009)	0.11 (0.006)	<0.001	<0.05	<0.05
Stems	0.49 (0.006)	0.50 (0.015)	0.45 (0.015)	0.45 (0.006)	<0.01	NS	NS
Roots	0.17 (0.003)	0.16 (0.010)	0.15 (0.017)	0.16 (0.012)	NS	NS	NS
Ears	0.13 (0.000)	0.13 (0.003)	0.15 (0.006)	0.16 (0.009)	<0.01	NS	NS
Dead	0.13 (0.003)	0.13 (0.007)	0.10 (0.007)	0.12 (0.009)	<0.05	NS	NS

*Final Harvest:*

CO <sub>2</sub>	Low N		High N		F Probability		
	36	70	36	70	N	CO <sub>2</sub>	N x CO <sub>2</sub>
Straw	0.39 (0.008)	0.41 (0.011)	0.35 (0.006)	0.35 (0.007)	<0.001	NS	NS
Root	0.26 (0.004)	0.25 (0.006)	0.28 (0.003)	0.28 (0.004)	<0.001	NS	NS
Grain	0.35 (0.004)	0.34 (0.006)	0.37 (0.002)	0.38 (0.004)	<0.001	NS	NS

As in Experiment One, at anthesis low N grown plants contained a significantly smaller ( $P<0.001$ ) fraction of crop dry mass in their leaves, with significantly less ( $P<0.05$ ) at elevated CO<sub>2</sub>, these effects having a significant ( $P<0.05$ ) interaction (Table 2.8).

Compared to low N, the high N grown plants of Experiment Three had significantly smaller ( $P<0.05 - 0.001$ ) fractions of crop dry mass allocated to stems and dead leaves at anthesis, and then straw at final harvest, but with significantly greater ( $P<0.01 - 0.001$ ) dry mass allocation to ears at anthesis and grain at final harvest (Table 2.8). Presumably again, this reflects an earlier or faster remobilisation of resources, because more grains produced at high N (Table 2.9 below) will generate a proportionately stronger sink than is the case for the low N plants. Carbon dioxide enrichment however, did not affect any of these patterns of response at either harvest.

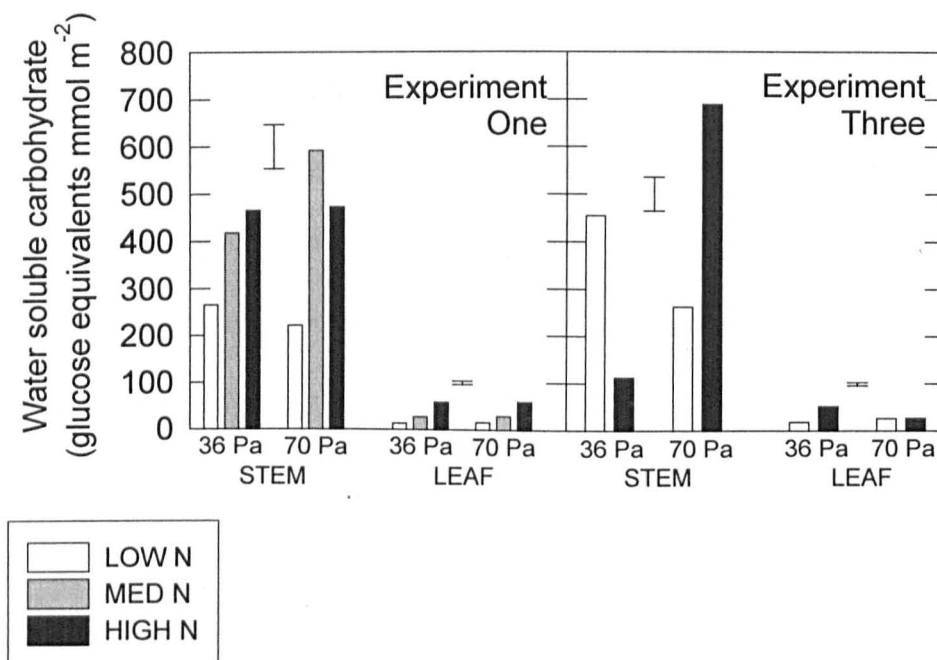
Grain yield (Table 2.9) was affected by N and CO<sub>2</sub> in an extremely similar manner to biomass at final harvest in Experiment One, with CO<sub>2</sub> stimulations of 6, 10 and 19% for the low, medium and high N treatments respectively.

N treatment		Low		Medium		High		F probability		
CO <sub>2</sub> (Pa)	Expt	36	70	36	70	36	70	N	CO <sub>2</sub>	N.CO <sub>2</sub>
No. ears	1	243	243	480	486	707	723	<0.001	NS	NS
(m <sup>-2</sup> )	3	477	673			495	753	<0.001	<0.01	<0.01
No. grains	1	6.2	6.7	13.6	14.6	23.3	27.0	<0.001	<0.01	<0.05
(10 <sup>3</sup> m <sup>-2</sup> )	3	14.3	15.3			24.7	28.2	<0.001	<0.001	<0.05
Grain yield	1	201	213	422	463	722	849	<0.001	<0.01	<0.01
(g m <sup>-2</sup> )	3	492	518			739	861	<0.001	<0.001	<0.01
Mean grain mass (mg)	1	32.2	32.1	31.0	31.8	30.8	32.4	NS	NS	NS
No. of grains/ear	1	26.0	27.5	28.4	30.8	33.0	36.6	<0.001	<0.05	NS

**Table 2.9** Treatment effects on grain yield and components at final harvest for Experiments One and Three. Results are from CO<sub>2</sub> x N ANOVA, where n=8.

However, in Experiment Three, the CO<sub>2</sub> stimulation was slightly less for grain yield than for total biomass (5% and 16% for low and high N treatments respectively). This is explicable in terms of the difference between experiments in N treatment regimes (Fig. 2.1). In Experiment Three, applications stopped earlier so that N limitation was at its most severe at the end of the season, so that the CO<sub>2</sub> stimulation would be less during this period. Later growth has proportionately greater effect on grain yield than early growth in wheat (Mitchell et al., 1996). Nevertheless, the observed stimulation of yield at 70 Pa CO<sub>2</sub> for the high N grown plants of this study, are comparable to the results of others for wheat grown at 70 Pa CO<sub>2</sub> and ample N (Havelka et al., (1984) 17% increase at 120 Pa CO<sub>2</sub>; Mitchell et al., (1993) 15% stimulation; Mitchell et al., (1996) 13%; Mulholland et al., (1997) 33% increase).

The effect of CO<sub>2</sub> enrichment on grain yield was attributable to an increase in the number of grains per ear for plants of Experiment One (Table 2.9). In Experiment Three, the CO<sub>2</sub> enrichment effect on yield was due to increases in both the number of grains per ear and the number of ears, although the former component was of more significance than the latter (Table 2.9). In line with Mulholland et al., (1997), mean grain mass (Table 2.9) was not affected by either N or CO<sub>2</sub> treatment, supporting the argument that there is a genetically determined upper limit to grain size which is unaffected in CO<sub>2</sub> enriched plants (Lawlor and Mitchell, 1991).

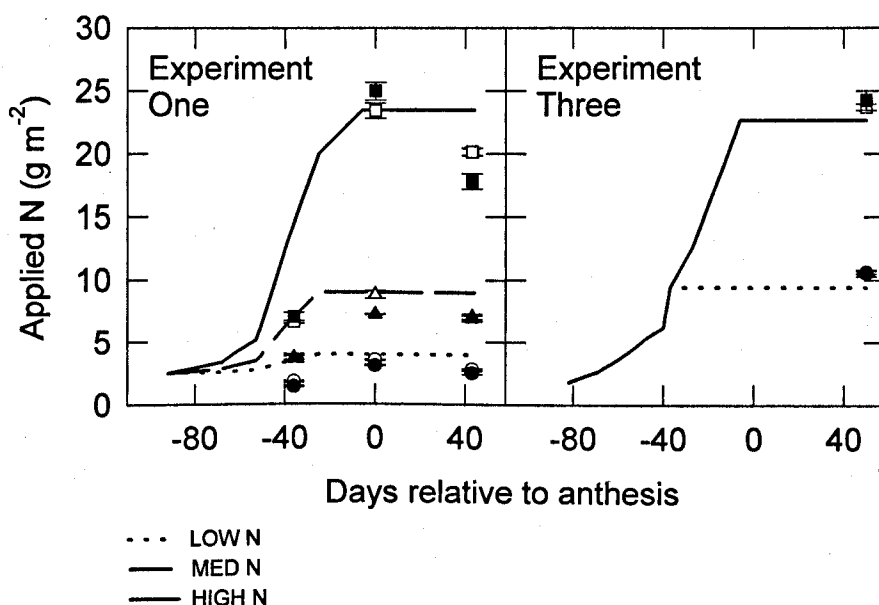


**Figure 2.9** Water soluble carbohydrate content in leaves and stems measured at anthesis in Experiments One and Three. Bars = 1x SED from CO<sub>2</sub> x N ANOVA, where n=4.

The water-soluble carbohydrate concentration in leaves and stems for both experiments at anthesis (Fig. 2.9), increased significantly ( $P<0.05$ ) with N, except for the ambient grown stems in Experiment Three. Enrichment with CO<sub>2</sub> had no significant effect on water-soluble carbohydrate content in leaves and stems of plants in Experiment One, and was only significant ( $P<0.01$ ) in Experiment Three for the ambient CO<sub>2</sub> grown stems.

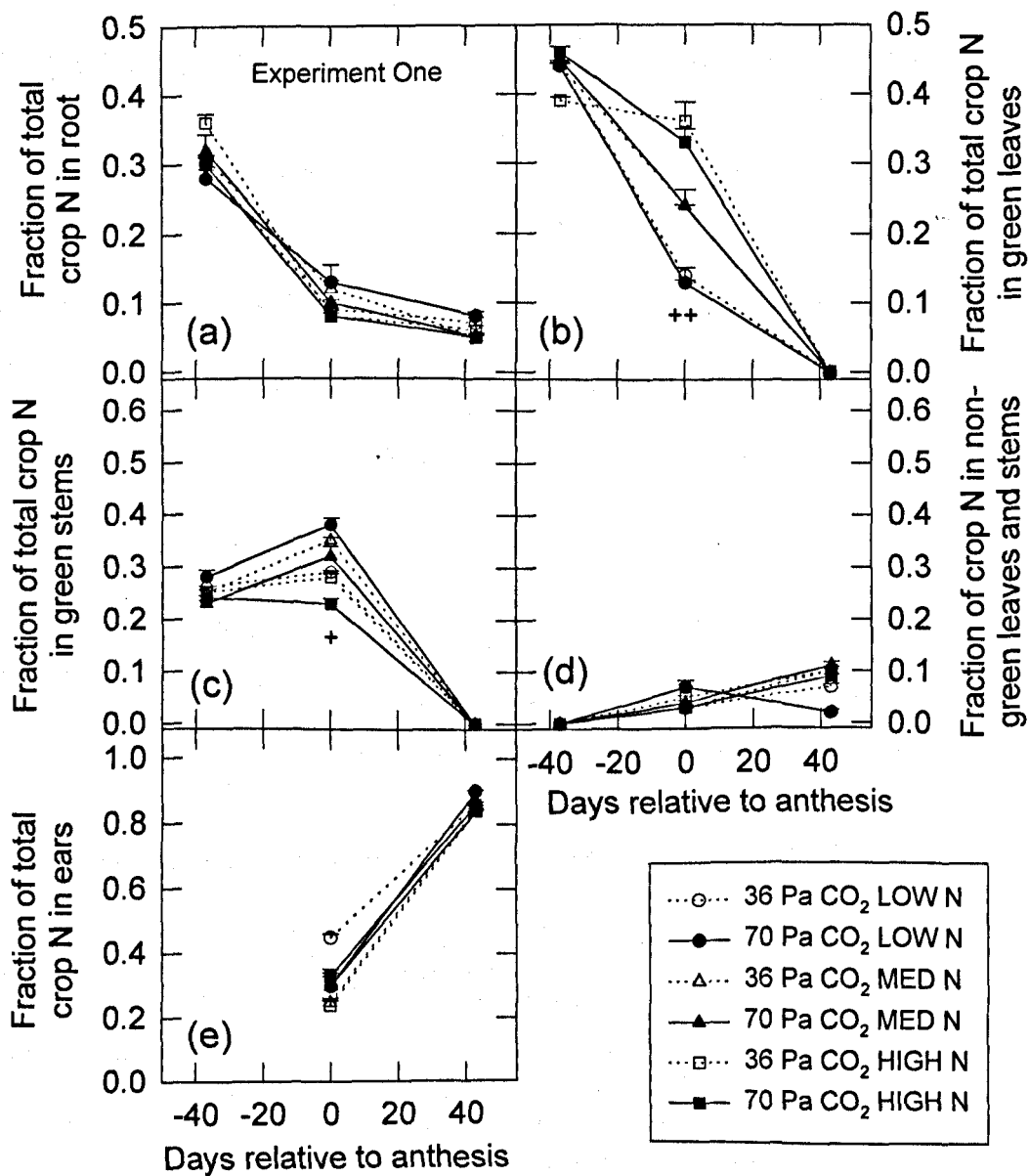
In both experiments, total crop uptake of N was unaffected by CO<sub>2</sub> and all of the applied N was taken up by anthesis in Experiment One (Fig. 2.10). Between anthesis and final harvest about 30% of the N was apparently lost from the crop in Experiment One, but not in Experiment Three.





**Figure 2.10** Nitrogen applied (lines) and total crop N uptake (symbols) over time. Error bars represent  $\pm$  SE of 6-8 replicates. Symbols as in Figure 2.8.

The fraction of total N in green leaves at anthesis in Experiment One was significantly affected by N treatment ( $P < 0.01$ ) with values of 0.14, 0.24 and 0.35 going from low to high N treatments (Fig. 2.11 b), this being at an increased cost to the fraction of N allocated to stems, having values of 0.38 to 0.23 going from low to high N treatments (Fig. 2.11 c). However, on no occasion for this experiment, was there any significant effect of N or CO<sub>2</sub> on the allocation of N to any other plant part (Fig. 2.11).



**Figure 2.11** Fraction of total crop N distributed to different plant parts, for the three harvests of Experiment One. Asterisks denote significant effects of CO<sub>2</sub> for the occasion indicated: \* = ( $P < 0.05$ ), \*\* = ( $P < 0.01$ ) and \*\*\* = ( $P < 0.001$ ). Addition signs denote significant effects of N for the occasion indicated: + = ( $P < 0.05$ ), ++ = ( $P < 0.01$ ) and +++ = ( $P < 0.001$ ).

**Table 2.10** Fraction of total crop N distributed to different plant parts, for the final harvest of Experiment Three. Values are the means of four replicate pots, each pot containing nine plants. Values in parentheses are 1 x SE.

*Final Harvest:*

CO <sub>2</sub>	Low N		High N		F Probability		
	36	70	36	70	N	CO <sub>2</sub>	N x CO <sub>2</sub>
Straw	0.33 (0.032)	0.34 (0.015)	0.26 (0.050)	0.23 (0.026)	<0.05	NS	NS
Root	0.12 (0.023)	0.16 (0.017)	0.05 (0.005)	0.08 (0.007)	<0.001	NS	NS
Grain	0.55 (0.034)	0.50 (0.020)	0.69 (0.051)	0.70 (0.028)	<0.001	NS	NS

In contrast, for the final harvest of Experiment Three (Table 2.10), whilst as for Experiment One, CO<sub>2</sub> treatment had no significant effect on N allocation to plant parts, N treatment resulted in significantly more N being retained in straw ( $P<0.05$ ) and root ( $P<0.001$ ) for low N grown plants, at a cost to significantly less ( $P<0.001$ ) N being remobilised to the grain.

As discussed above, in Experiment One, the fraction of total crop N allocated to the grain was not affected by N or CO<sub>2</sub> treatments. As a consequence of this, the percentage N content of grain (Table 2.11) was significantly increased ( $P<0.001$ ) with N, and significantly decreased ( $P<0.001$ ) with CO<sub>2</sub>. However, in Experiment Three, the fraction of N in the grain was decreased at lower N treatment, as more of it was sequestered in vegetative tissue from which it could not be re-mobilised (Table 2.10). Furthermore, a slight decrease in %N of grain with elevated CO<sub>2</sub> was not significant in Experiment Three (Table 2.11). The results emphasise the importance of differences in timing of N applications. The generally earlier applications in Experiment Three were much less efficiently re-mobilised to the grain compared with Experiment One, and the efficiency was further decreased in the Experiment Three low N treatment, where N application stopped earlier still (Fig. 2.1).

N treatment		Low		Medium		High		F probability		
CO <sub>2</sub> (Pa)	Expt	36	70	36	70	36	70	N	CO <sub>2</sub>	N.CO <sub>2</sub>
Grain %N	1	1.15	0.99	1.44	1.19	2.42	1.92	<0.001	<0.001	NS
	3	1.09	1.04			2.20	2.01	<0.001	NS	NS

**Table 2.11** Treatment effects on grain N at final harvest for Experiments One and Three. Results are from a CO<sub>2</sub> x N ANOVA, n =8.

In summary, growth of spring wheat at elevated CO<sub>2</sub> in two separate experiments, did not affect phenology or mainstem leaf appearance, but increased biomass and grain yield by about 5% at low N and 20% at high N. This interaction was consistently observed in both experiments. The effect on yield was almost entirely due to increased number of grains. There was no consistent increase in fraction of dry mass in roots at elevated CO<sub>2</sub> and low N. Total N uptake was unaffected by CO<sub>2</sub>, and there was no decrease in the fraction of crop N in green leaves at elevated CO<sub>2</sub> up until anthesis. However, following grain-fill, the fraction of crop N in the grain at final harvest was not significantly greater. In general therefore, there was no evidence for a substantial redistribution of N away from leaves to other organs, even under conditions of a severely limiting N supply. This suggests that at the whole plant level, spring wheat does not respond to elevated CO<sub>2</sub> in the optimal manner to maximise yield under deficient N conditions.



## CHAPTER 3

### The long-term effect of elevated partial pressures of CO<sub>2</sub> on the *in vivo* balance between capacities determining photosynthesis in wheat

#### 3.1 Introduction

Leaf gas exchange analysis is a non-destructive tool which can be used to assess, amongst other environmental factors, the effect of changes in  $pC_a$  on the *in vivo* balance between component capacities which determine photosynthetic rate (Sharkey, 1985; Sage et al., 1989). Typically the response of net carbon assimilation at saturating light intensity ( $A$ ) versus intercellular CO<sub>2</sub> partial pressure ( $pC_i$ ) is measured, as such analyses avoid the potential limitations to photosynthetic rate from changes in stomatal conductance. Therefore observations relate directly to the underlying biochemistry, albeit modified by the resistance to CO<sub>2</sub> transfer from intercellular spaces to sites of carboxylation, as discussed below.

As discussed in Chapter One, the  $A/pC_i$  response is related to the photosynthetic biochemistry in terms as defined by the steady-state model of C<sub>3</sub> photosynthetic carbon metabolism (von Caemmerer and Farquhar, 1981; Farquhar and Sharkey, 1982), these being that (1), the initial linear slope of the  $A/pC_i$  response at low  $pC_i$  relates to the quantity of active Rubisco, so determining the carboxylation capacity, (2) that the response at high  $pC_i$  reflects the capacity of enzymes of electron transport, photophosphorylation (i.e. the thylakoid ATP-synthase) and PCR enzymes both in amount and activation to drive the regeneration of RuBP and hence is termed the RuBP-regeneration capacity, and (3) that the asymptote at saturating  $pC_i$  can sometimes reflect a limitation by the capacity of starch and sucrose synthesis to regenerate ortho-phosphate (PO<sub>4</sub><sup>3-</sup>) for

photophosphorylation, and is termed the  $\text{PO}_4^{3-}$  regeneration capacity (Sharkey, 1985; Stitt, 1986). According to the theory of Sage et al. (1988) these two latter capacities can be distinguished, as  $\text{PO}_4^{3-}$  regeneration limited photosynthesis is insensitive to further increases in the concentration of  $\text{CO}_2$  or decreasing  $\text{O}_2$ , whereas photosynthesis limited by RuBP-regeneration capacity is stimulated further by the same changes in these respective gases.

As detailed in Chapter One, at current  $pC_a$  it is considered that these capacities are usually in balance and equally co-limiting, so that photosynthesis is maximised and the allocation of resources into components which determine rate are fully optimised. However, with  $\text{CO}_2$  enrichment the efficiency of the carboxylation process will improve substantially, resulting in a shift in the *in vivo* balance between capacities, and a new limitation to photosynthesis imposed by either one of RuBP or  $\text{PO}_4^{3-}$  regeneration capacity, or indeed shared by both. That a new limitation to photosynthetic rate is imposed by RuBP and/or  $\text{PO}_4^{3-}$  regeneration capacity, is evidenced by the fact that for a doubling of  $pC_a$  to 70 Pa, rates of photosynthesis in the short-term are only generally stimulated by about 50% (Cure and Acock, 1986), which is well below the theoretical stimulation of 110% when it is assumed that RuBP remains saturating (calculation in Stitt, 1991; Makino, 1994). However, in the long-term, theory suggests, based on observed responses to other environmental factors such as irradiance and temperature (section 1.4.3), that in order to maintain efficiency this imbalance between *in vivo* capacities could be redressed in one of several ways which have been discussed previously (section 1.4). Assuming that re-balancing of *in vivo* capacities does occur in response to  $\text{CO}_2$  enrichment, then a change in the relative ratios of one capacity to another should be



detectable by applying gas exchange techniques and looking for a change in the shape of the  $A/pC_i$  response curve.

To date, evidence suggestive of a rebalancing of *in vivo* capacities in response to CO<sub>2</sub> enrichment, has been limited to a minority of studies done on potato (Sage et al., 1989), *Castanea sativa* (Mousseau and Saugier, 1992) and wheat (Hicklenton and Joliffe, 1980). These tended to be in studies using young plants (*Castanea sativa*), low light intensities during growth (wheat), or plants with large storage capacities (potato). Conversely, in an analysis of over 40 studies investigating the long-term effects of elevated CO<sub>2</sub> on the  $A/pC_i$  response, Sage (1994), concluded that the response observed was more dependent on growth conditions than CO<sub>2</sub> treatment, as plants grown in small pots or at limiting nutrition, typically demonstrated an acclimation response with decreased  $A$  at all  $pC_i$ . Indeed, when pot size was not a factor, as for field grown plants, changes in the shape of  $A/pC_i$  curves were not observed, indicating that no decreases in either all components, or rebalancing between any *in vivo* capacities had taken place.

However, Delgado et al., (1994) grew stands of winter wheat at 35 and 70 Pa CO<sub>2</sub> and at low and high N applications, and for 70 Pa CO<sub>2</sub> grown plants observed a 10% mean increase in photosynthetic rate over the whole experiment for both N treatments, whilst there were no differences in carboxylation efficiency derived from the initial slope of the  $A/pC_i$  response, and indicative of the amount of fully activated Rubisco which ultimately determines carboxylation capacity. This observation suggests that there may have been relative increases in the *in vivo* capacities for RuBP and/or PO<sub>4</sub><sup>3-</sup> regeneration over carboxylation capacity, so redressing to some degree, the balance at 70 Pa CO<sub>2</sub> in accordance with theory,

although the effect was only just statistically significant in their data.

Thus, the aim of the work described in this chapter was to determine whether long-term growth of spring wheat at 70 Pa CO<sub>2</sub> in three experiments, and 100 Pa CO<sub>2</sub> in a fourth, would have any effect on the shape of the  $A/pC_i$  responses from leaves, (i.e. specifically a decrease in the initial slope relative to the asymptote) and hence the relative ratios between *in vivo* capacities. Additionally, the effect on the *in vivo* balance between the capacities of RuBP and PO<sub>4</sub><sup>3-</sup> regeneration was assessed by measuring the O<sub>2</sub> sensitivity response.

In this chapter,  $A/pC_i$  data are presented with the minimum of interpretation, without taking into account the possibility of a substantial and variable transfer resistance, as is frequently done (Besford et al., 1990; van Oosten et al., 1995). In Chapter Six, where we relate gas exchange parameters to components, the importance of this variable will be addressed.

In the following pages, the method of gas exchange analysis is described, followed by the presentation of various parameters derived from  $A/pC_i$  responses, expressed first on a leaf area basis as a function of time, and then on a leaf area basis as a function of leaf N content. Finally, conclusions will be drawn as to whether or not there was a long-term effect of elevated CO<sub>2</sub> on the  $A/pC_i$  response and balance between *in vivo* capacities determining photosynthesis.

### 3.2 Materials and Methods

The response of light-saturated leaf  $A$  to varying  $pC_i$  was determined in a six-chamber open circuit gas exchange system with automatic data handling (Lawlor et al., 1989), using principles and techniques discussed by Long and Hallgren (1993).

The system consisted of mode switching (WA-357-MK3; ADC, Hoddesdon, Herts, UK) and gas-handling units (WA-161 2 K; ADC), with the CO<sub>2</sub> partial pressure and partial pressure difference during experiments being measured by an IRGA (WA-255-MK3; ADC), calibrated regularly during experiments in both absolute and differential mode, and frequently cross checked with standard gas mixtures (Air Products, Thetford, Norfolk, UK). The O<sub>2</sub> partial pressure was measured by a portable gas analyser (Series 80; Ox-An Systems, Huddersfield, UK), with variable CO<sub>2</sub> and O<sub>2</sub> partial pressures being generated by a gas blender (Signal Instruments Co., Croydon, UK) against N<sub>2</sub> used as a carrier gas, and reflective of atmospheric composition. The mass flow of the resulting mixture was measured with mass flow meters and controllers (Bronkhorst HI-TECH B.V., Ruurlo, Holland), and the gas stream was humidified by passage through a bubbler set at 20°C, and followed by a condenser set to the required dew point such that the vapour pressure deficit between the leaf and chamber air ranged from 1 to 1.5 kPa. The humidity of air before and after passage over the leaf was determined with capacitance sensors (Vaisala, Helsinki, Finland), calibrated against a water vapour generator (WG-601; ADC). Actinic light with a PPFD of 1750  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$  was provided homogeneously across whole plants and the specific leaves under measurement, from metal-halide photoflood lamps (Wotan, Phillips, Holland). The PPFD at the surface of each leaf was measured with selenium sensors (Megatron, London, UK) calibrated against a quantum sensor (LI-189; Li-Cor-Inc., Lincoln, Nebraska, USA). Leaf temperature was calculated according to the energy budget of the leaf (Ehleringer, 1989), and measured with fine wire thermocouples in individual chambers and close (within 2 mm) to the lower surfaces of leaves. Temperature was

maintained by adjusting the temperature of individual leaf chamber water jackets in which leaves attached to plants were enclosed, defining a maximum leaf area window of 1000 mm<sup>2</sup>. Within leaf chambers, a gas flow of 90 mm<sup>3</sup> s<sup>-1</sup> was vigorously stirred to give a leaf boundary layer resistance of 0.35 m<sup>2</sup> s mol<sup>-1</sup>, determined according to Parkinson (1985).

It has previously been reported that there is a considerable ontogenetic gradient of development along the length of wheat leaves (Boffey et al., 1980), resulting in considerable variation in both photosynthetic rate and the activity/amount of biochemical components (confirmed in 5.2.1). Clearly this has the potential to introduce additional errors when expressing results on a leaf area basis, depending upon which part of leaves are specifically chosen and clamped within leaf gas exchange chambers. However, for a given supply of N during growth, wheat leaves tend to show little variation in length or area (section 2.8.3), such that potential sampling variation can be minimised if replicate leaves are clamped in the same measured positions; in these experiments the lamina section between 60 and 160 mm from leaf tips (since the chamber was 100 mm long) were clamped. Leaves were labelled to allow for subsequent re-measurement of the same leaf section later in the photoperiod (as for 3.2.3 below) or on a later date, provided that leaves were already fully expanded on the first occasion measured.

For the first three experiments, attached leaves of well-watered potted plants were selected and equilibrated for 10 minutes at a PPFD of 1750  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ , after which net photosynthesis was measured at 21 kPa O<sub>2</sub> and the growth  $pC_a$  specific to plants selected, i.e. 36 or 70 Pa CO<sub>2</sub>. When photosynthetic rate was constant, the  $A/pC_i$  response was determined at values of  $pC_i$  in the order of

approximately 2, 4, 7, 10, 14, 25, 50, 60 and 68 Pa for each leaf. Additionally and following measurement of the  $A/pC_i$  response, the sensitivity of leaf photosynthesis to changing  $O_2$  partial pressure from ambient (21 kPa) to 2 kPa was determined at  $pC_a=70$  Pa in Experiment Two, and  $pC_a=36$  and 70 Pa in Experiment Three. Leaf sections within chambers were then cut from the remainder of leaf lamina, placed in foil envelopes and stored in liquid  $N_2$  for the measurement of amounts of biochemical components (Chapters Four and Five).

For Experiment Four, plants were equilibrated at the specific growth  $pC_a$  of either 36 or 100 Pa  $CO_2$ , and then the  $A/pC_i$  response was determined at values of  $pC_i$  in the order of 2, 4, 10, 14, 20, 36, 68, 87 and 100 Pa for each leaf. Next, the sensitivity of leaf photosynthesis to changing  $O_2$  partial pressure from ambient (21 kPa) to 2 kPa was determined at  $pC_a=20$ , 36 and 100 Pa. Thereafter, leaves were returned to their growth  $pC_a$  and 21 kPa  $O_2$ , and allowed to equilibrate under the saturating PPFD of  $1750 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$  for a minimum of 10 minutes or until  $A$  was constant. Leaf sections within the chamber were then freeze-clamped within 0.2 s with a liquid  $N_2$  cooled clamping tool developed at IACR-Rothamsted. This operation was facilitated by virtue of gas exchange chambers having upper and lower surfaces of transparent "Clingfilm" and aluminium foil respectively, so permitting easy puncture by the clamping tool. Samples were then rapidly placed in foil envelopes and stored under liquid  $N_2$ , for the determination of initial, total and maximal activity of Rubisco (Chapter Four).

On average, it took three hours to determine the  $A/pC_i$  and  $O_2$  sensitivity responses described above for six leaves (three replicates of each growth  $CO_2$  treatment) during each gas exchange run. Therefore, on days where 18 separate

leaves were analysed (three runs), the last six leaves will have been exposed to an additional six hours of photoperiodic illumination as they stood in the growth environment. Azcón-Bieto (1983) demonstrated that the net CO<sub>2</sub> assimilation rate of mature wheat leaves in ambient air (34 Pa CO<sub>2</sub>) declined with time of illumination during the photoperiod, particularly at temperatures lower than 25°C, due to an accumulation of carbohydrates. Therefore, to eliminate the possibility of this effect causing underestimation of  $A/pC_i$  values (particularly at high  $pC_i$ ), three and a half hours before each gas exchange run was due to commence, selected plants within the growth environment were covered with black polythene bags supported on canes to eliminate actinic light. Bags were removed 30 minutes prior to transferring plants from the growth environment to the laboratory containing the gas exchange equipment, where plants were allowed to equilibrate for a length of time, such that analysis on all plants of all gas exchange runs, began one hour into the “new” photoperiod. Three hours of darkness was chosen, because Azcón-Bieto (1983) demonstrated that this was a sufficient length of time to relieve the depression on photosynthesis, in experiments where plants had been subjected to treatments far more severe than conditions used in this study.

### 3.2.1 *Calculation of photosynthetic parameters*

For the four experiments presented in this study, a total of 675 individual  $A/pC_i$  responses were determined. Therefore to make comparisons between the shape of curves, common parameters from each were derived. For individual leaf responses of all experiments, polynomial regressions were fitted (Genstat 5, Rothamsted Experimental Station), and the carboxylation efficiency ( $\epsilon$ ), which

reflects the amount of active Rubisco, was determined from the initial linear slope of curves at the  $pC_i$  corresponding to the  $\text{CO}_2$ -photocompensation point, at which gross photosynthesis exactly balances mitochondrial and photo-respiration, so that net photosynthesis equals zero (Farquhar et al., 1980).  $A_{\max}$  values, indicative of limitation by other capacities (typically RuBP-regeneration) were determined at the maximal value of  $A$  over the range of  $pC_i$  measured.

### 3.2.2 *Experiment to demonstrate the light saturation of $A/pC_i$ responses*

#### *Introduction*

In order that *in vivo* activities of leaf components are truly reflected in the photosynthetic response to changing  $pC_i$ , it is necessary that the PPFD provided during gas exchange analysis is saturating. To demonstrate that this was the case for the gas exchange system and the cultivar being used in this study, the following light response analysis was done.

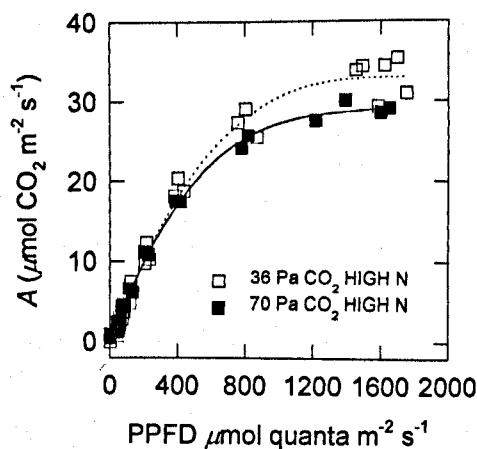
#### *Materials and method*

Flag leaves at full emergence from high N, 36 and 70 Pa  $\text{CO}_2$  grown plants of Experiment Two were selected and measured at  $20^\circ\text{C}$ , 21 kPa  $\text{O}_2$  and 70 Pa  $\text{CO}_2$  using the apparatus and other conditions described in section 3.2. After equilibration for 30 minutes and to check that leaves were photosynthetically competent, the PPFD was reduced to zero by placing a series of neutral density filters (Lee Filters; A.C Lighting, Bucks, U.K.) and finally a layer of black cloth, over plants and leaf samples within gas exchange chambers. Following 30 minutes in darkness, the black

cloth and filters were sequentially removed, to create a PPFD range from 0 to 1750  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ . At each PPFD, leaves were allowed to equilibrate for at least 15 minutes prior to measurement. Polynomial regressions were fitted to the data of each response (SigmaPlot v.2.0; Jandell Scientific, Germany), and the apparent quantum yield of  $\text{CO}_2$  fixation was calculated from the slope of the linear response of net assimilation ( $A$ )/PPFD at 40-130  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD.

### Results and discussion

The response of net assimilation to PPFD shown in Figure 3.1, demonstrates that the light intensity of the gas exchange system was indeed sufficient to saturate the photosynthetic response of the Minaret cultivar used in this study, specifically saturating 36 Pa  $\text{CO}_2$  grown plants at 1400  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ , and 70 Pa  $\text{CO}_2$  grown plants at approximately 1600  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ .



**Figure 3.1** Response of net  $\text{CO}_2$  assimilation ( $A$ ) to PPFD, in fully expanded flag leaves of spring wheat grown at high N application and the  $\text{CO}_2$  partial pressure denoted by the key. Measurements were made at  $20^\circ\text{C}$  and at 70 Pa  $\text{CO}_2$ , with three replicate leaves for each  $\text{CO}_2$  treatment. For further details see text.

Mean values for apparent quantum yield,  $A_{\text{sat}}$  and respiration in the dark ( $R_d$ ) were not significantly affected by growth  $\text{CO}_2$  (Table 3.1), and absolute values



obtained are comparable to those previously reported elsewhere for this species (Habash et al., 1989). Thus it was concluded that  $A/pC_i$  responses were indeed sufficiently light saturated.

	$pC_a$ during growth (Pa)	
	36	70
Maximum apparent quantum yield ( $\mu\text{mol CO}_2 \mu\text{mol}^{-1}$ PPFD)	0.054 (0.002)	0.050 (0.002)
$A_{\text{sat}}$ ( $\mu\text{mol CO}_2 \text{m}^{-2} \text{s}^{-1}$ )	33.0 (2.15)	28.7 (0.30)
$R_d$ ( $\mu\text{mol CO}_2 \text{m}^{-2} \text{s}^{-1}$ )	-0.372 (0.32)	-0.008 (0.007)

**Table 3.1** Photosynthetic parameters (derived from the curves shown in Fig. 3.1) of fully emerged flag leaves grown at high N and either 36 or 70 Pa  $\text{CO}_2$ , for which the responses of photosynthesis ( $A$ ) to varying PPFD were determined at 20°C and saturating (70 Pa)  $\text{CO}_2$ . SE values are shown in parentheses, and  $n = 3$ .

Further, since recently fully emerged, high N, 36 and 70 Pa  $\text{CO}_2$  grown plants had been tested at 70 Pa  $\text{CO}_2$ , which would be expected to give the highest photosynthetic rates, it is reasonable to assume that any subsequent older leaf grown at a lower N application, would equally be light saturated in its  $A/pC_i$  response.

### 3.2.3 *Experiment to determine whether photosynthetic rates at high $pC_i$ decline through the photoperiod in wheat*

#### *Introduction*

As reported above, Azcón-Bieto (1983) demonstrated that the net  $\text{CO}_2$  assimilation rate of mature wheat leaves in ambient air (34 Pa  $\text{CO}_2$ ) can decline with time of illumination during the photoperiod, and particularly at temperatures lower than 25°C. Since plants were grown and measured at 20°C in this study, precautions against such an effect were taken and have been discussed. However, as the

response is an interesting one, and to determine whether the precautions were really necessary, the principle experiment of Azcón-Bieto (1983) was repeated.

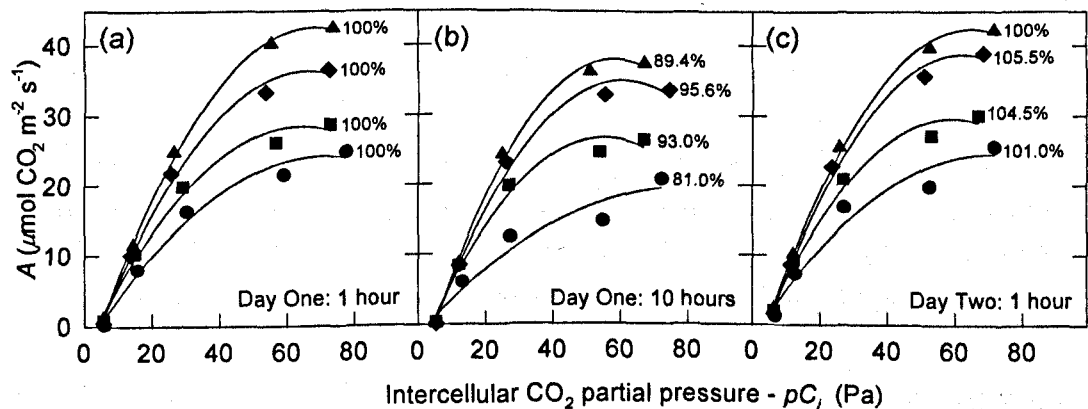
### *Materials and methods*

The  $A/pC_i$  response was determined at 6, 16, 35, 70 and 90 Pa, at 20°C and 21kPa O<sub>2</sub> in light saturated, high N and 36 Pa CO<sub>2</sub> grown flag leaves at full emergence from Experiment Two, initially at one hour into the photoperiod of, for the purposes of this experiment, day one. All other conditions for analysis were as described previously. Following the determination of the  $A/pC_i$  response, which due to the fewer points measured took approximately an hour less than normal, measured leaves were labelled and the plants to which they were attached were returned to the growth environment for a further seven hours of exposure to the photoperiod. The  $A/pC_i$  responses for the same leaves, clamped in the same positions, were then determined at the start of hour 10 of the photoperiod (which includes the two hours of illumination during the previous analysis). Thereafter, plants were returned to the growth environment, where they were exposed to the photoperiod for a further two hours (to give a total of 14 hours for the day), before a 10 hour dark period. Plants were then measured a third and final time, one hour into the photoperiod of day two. Responses were fitted with polynomial regressions (SigmaPlot, Jandell Scientific, Germany), and  $A_{\max}$  determined at the maximal value of  $A$  over the range of  $pC_i$  measured.

### *Results and discussion*

The  $A/pC_i$  response in flag leaves before and after a long period of

photosynthesis under growth conditions are shown in Figure 3.2. Initial slopes were unaffected, and this was anticipated, for which reason few low  $pC_i$  points were measured. However, as Azcón-Bieto (1983) observed, the saturated region of curves at high  $pC_i$  ( $A_{\max}$ ) were on average significantly decreased ( $P<0.01$ ) to 89.8% ( $\pm 3.3$  SE) of that at the start of the photoperiod following 10 hours of photosynthesis under growth conditions (Fig 3.2 b).



**Figure 3.2** The effect of a period of photosynthesis through the day under growth conditions, on the  $A/pC_i$  response in flag leaves of wheat at full emergence, grown at a high N supply and 36 Pa  $CO_2$ . The  $A/pC_i$  response was determined in the same leaves at 1 hour into the photoperiod (a), 10 hours into the photoperiod (b), and 1 hour into the photoperiod the following day after a 10-hour dark period. Responses for four replicate leaves are shown, for which different symbols are used to aid interpretation. Values shown are the percentage  $A_{\max}$  of the  $A_{\max}$  obtained at 1 hour on day one, for each replicate leaf. For further details see text.

However, following a 10 hour period of darkness, this depression of photosynthesis was on average fully reversed, and to slightly beyond, the value obtained 24 hours previously ( $102.8\% \pm 1.4$  SE; Fig 3.2 c). These results are consistent with those of Azcón-Bieto (1983), for which he demonstrated and concluded that the accumulation of carbohydrates within leaves was responsible for the end product inhibition of photosynthesis, and most likely due to restricted translocation at temperatures below  $25^\circ\text{C}$ . Indeed, above a critical threshold leaf

carbohydrate concentration of 100 mmol C m<sup>-2</sup>, the depression of assimilation started and was found to be closely correlated with carbohydrate concentration (Azcón-Bieto, 1983; Fig. 3 therein).

In conclusion, photosynthesis in leaves of wheat used in this study and measured at high  $pC_i$  was susceptible to a small depression through the photoperiod, and therefore the precautions taken to safeguard against this were justified. Additionally, the fact that the depression in  $A_{\max}$  was fully recovered at the beginning of the photoperiod on day two, demonstrates that the method of clamping and containing leaves within chambers for periods of up to two hours was not deleterious to photosynthetic performance, and that the calibration and use of the gas exchange equipment was consistent and reliable.

#### 3.2.4 *Criteria for photosynthesis measurements in Experiments One to Four*

In Experiment One,  $A/pC_i$  measurements were made at a leaf temperature of 18(±0.5)°C as representative of the U.K. field environment, but following a decision to move closer to the conditions under which the majority of measurements in the literature have been made, measurements were subsequently taken at 20(±0.5)°C in Experiments Two to Four. Additionally, this temperature was the same as the daytime growth temperature in the controlled environment growth facility. Responses in all experiments were determined in six replicate leaves for each treatment and occasion. In Experiment One, responses were determined in flag leaves on four occasions, in Experiment Two in flag-1 and flag leaves on three and four occasions respectively, in Experiment Three in flag-2, flag-1 and flag leaves on three, three and six occasions respectively, and in Experiment Four, in flag leaves on

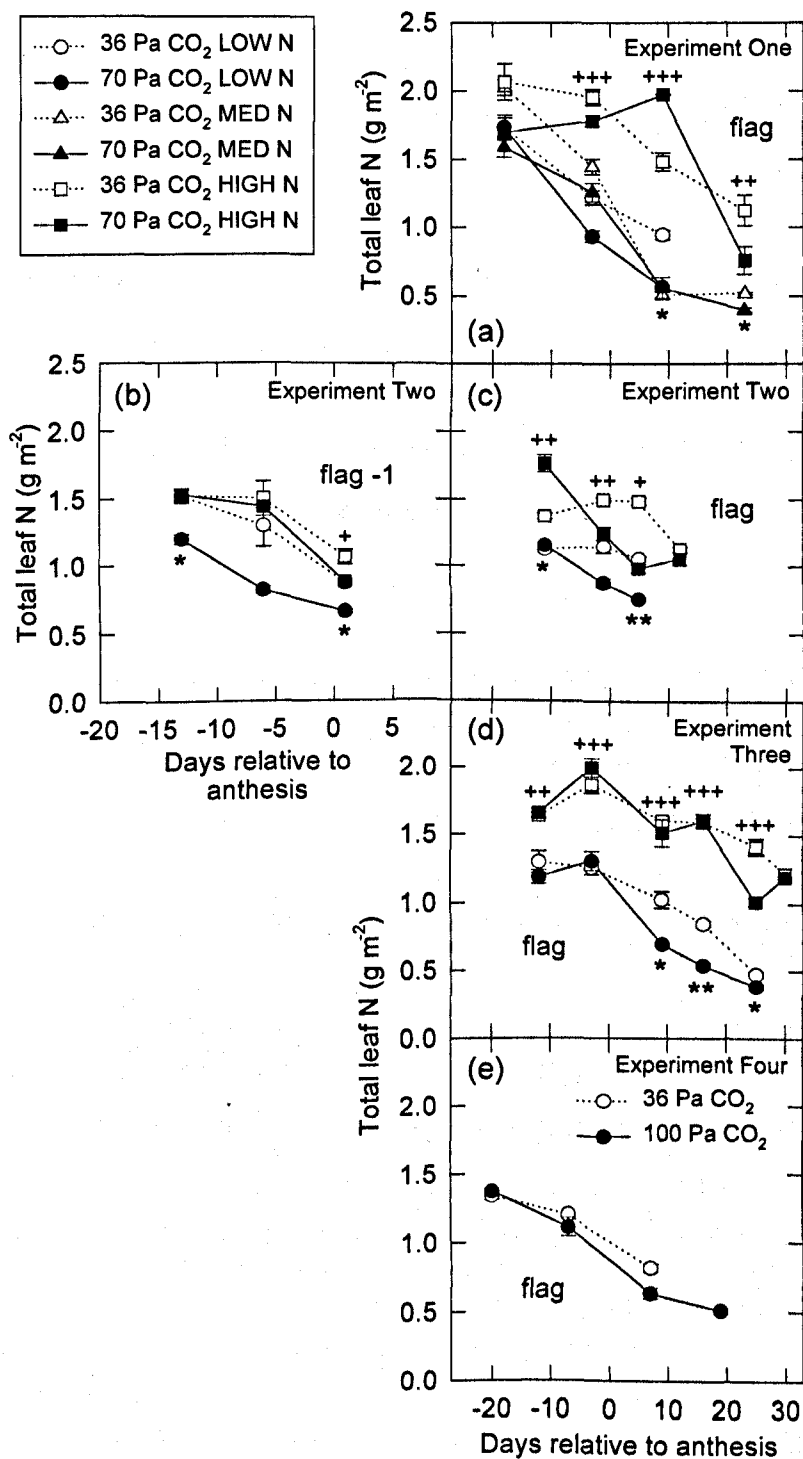
seven occasions. The first measurement occasion of each leaf coincided with ligule just visible. The total leaf N content of a selection of replicate samples from various leaves collected following gas exchange analysis, was determined using the method described in section 2.7, and these results are presented first.

### **3.3 Results: Responses of leaf N content, parameters derived from $A/pC_i$ curves and the $O_2$ sensitivity of photosynthesis to long-term growth at 70 Pa $CO_2$ as a function of time**

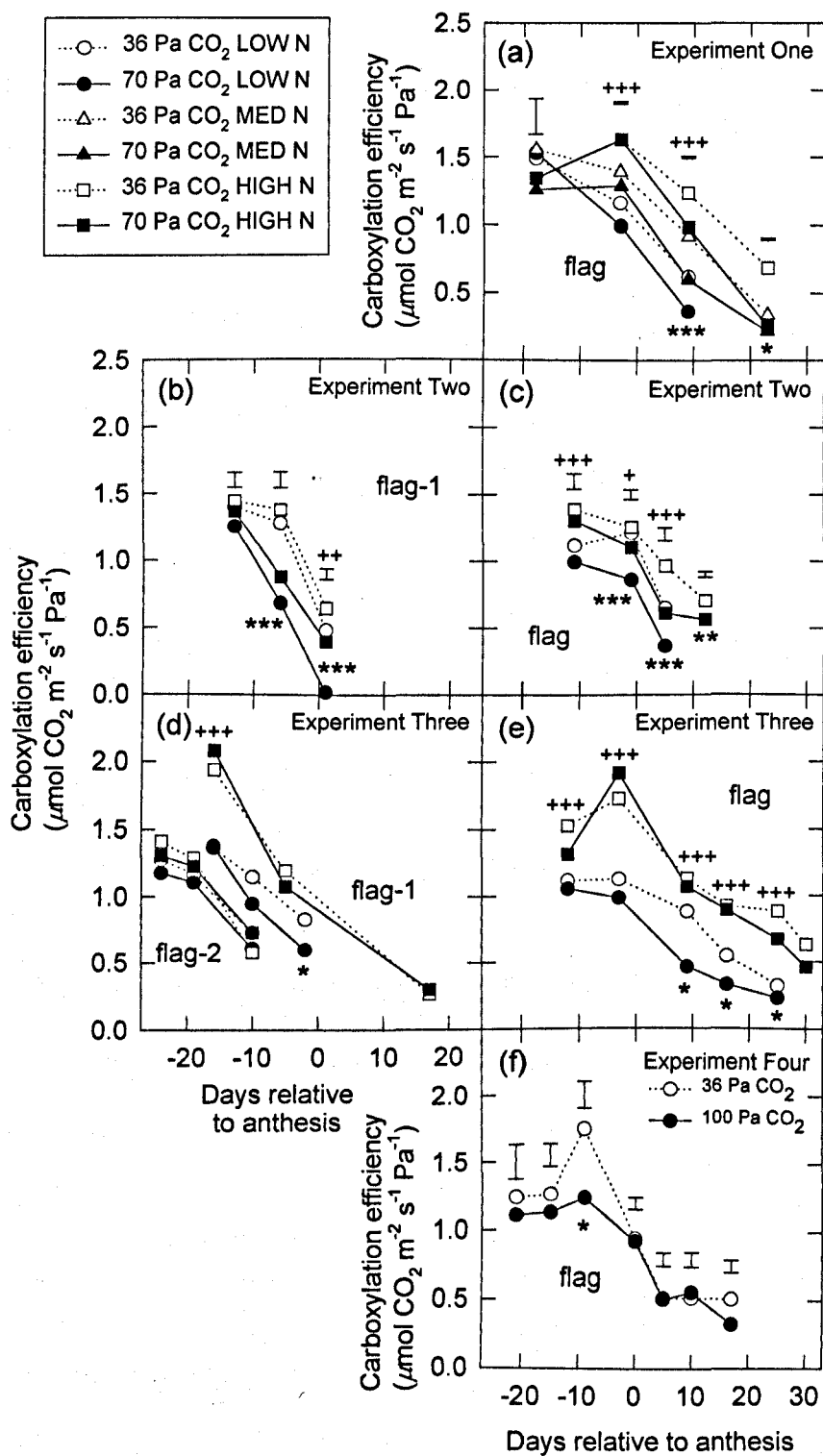
Regardless of specific treatments, and for all leaves of all experiments, absolute values of leaf N content showed a general pattern of decline with time as leaves aged (Fig. 3.3 a-e). As one might expect, the amount of N applied during growth significantly affected leaf N content in all leaves of all experiments, except for the first occasion in flag leaves of Experiment One (Fig. 3.3 a) and first and second occasions in flag-1 leaves of Experiment Two (Fig. 3.3 b). Where significant effects of N treatment were seen, not surprisingly, lower N applications during growth resulted in lower absolute leaf N contents (clearly illustrated in Fig. 3.3 d), which since this order within occasions was generally maintained through time, resulted in earlier leaf death first in low then medium and finally high N grown plants. Importantly, whilst different N treatments applied during growth exerted significant effects on leaf N content in flag leaves of experiments One to Three, the absolute differences between leaf N contents of high and low N treatments was far greater in flag leaves of Experiment Three (Fig. 3.3 d), than in flag leaves from Experiment Two (Fig 3.3 c). In flag leaves of Experiment One (Fig. 3.3 a) the differences in absolute leaf N contents between plants grown at low and medium N applications were not that different, whilst the

differences in absolute values between these treatments and high N grown plants were more comparable with flag leaves of Experiment Three.

Superimposed upon these patterns of decline in leaf N content, dependant upon the amount of N applied during growth, were significant effects of CO<sub>2</sub> enrichment. In low N grown flag-1 leaves of Experiment Two (Fig. 3.3 b), CO<sub>2</sub> enrichment caused significant ( $P<0.05$ ) decreases in leaf N content of plants grown at 70 Pa CO<sub>2</sub>, both at 13 days prior and one day after anthesis. For flag leaves, with the exception of a significant effect ( $P<0.05$ ) of CO<sub>2</sub> on leaf N content of high N grown flag leaves of Experiment Two at 11 days prior to anthesis (Fig 3.3 c) in which unexpectedly, leaf N content was greater in leaves of 70 Pa CO<sub>2</sub> grown plants, there was no significant effect of CO<sub>2</sub> on leaf N content in flag leaves, of any N treatment, experiment or occasion prior to five days after anthesis. Thereafter, significant effects of CO<sub>2</sub> enrichment on leaf N content were generally seen on all subsequent occasions, except in Experiment Four. Where significant CO<sub>2</sub> enrichment effects occurred for a given N treatment, leaf N contents were almost always lower in 70 compared to 36 Pa CO<sub>2</sub> grown leaves, and the magnitude of differences were generally greater for leaves of low compared to high N grown plants as is clearly illustrated in Fig 3.3d at 9 to 16 days after anthesis. There was no interaction between N and CO<sub>2</sub> on leaf N content, in any leaf of any occasion or experiment.



**Figure 3.3** Total leaf N content versus time in flag-1 (b) and flag leaves (a, c, d & e) of spring wheat grown in four separate experiments, at either 36 or 70 Pa CO<sub>2</sub> (Experiments One to Three, panels a-d), and 36 or 100 Pa CO<sub>2</sub> (Experiment Four, panel e). Nitrogen applications during growth are indicated in the key. Bars are 1 x SE, with n = 6 for Experiments One to Three, and n = 3 in Experiment Four. Asterisks denote significant effects from CO<sub>2</sub> x N ANOVAs of CO<sub>2</sub> for the occasion indicated: \* = (*P* < 0.05), \*\* = (*P* < 0.01) and \*\*\* = (*P* < 0.001), and plus signs denote significant effects of N for the occasion indicated: + = (*P* < 0.05), ++ = (*P* < 0.01) and +++ = (*P* < 0.001). For further details see text.



**Figure 3.4** Carboxylation efficiency versus time in flag-2 (d), flag-1 (b & d) and flag leaves (a, c, e & f) of spring wheat grown in four separate experiments, at either 36 or 70 Pa  $\text{CO}_2$  (Experiments One to Three, panels a-e), and 36 or 100 Pa  $\text{CO}_2$  (Experiment Four, panel f). Nitrogen applications during growth are indicated in the key. Bars are 1 x SED from  $\text{CO}_2 \times \text{N}$  ANOVA, with  $n = 6$  for each data point, except in Experiment Four, where bars are SED from  $\text{CO}_2$  ANOVA. Asterisks denote significant effects of  $\text{CO}_2$  for the occasion indicated: \* = ( $P < 0.05$ ), \*\* = ( $P < 0.01$ ) and \*\*\* = ( $P < 0.001$ ). Plus signs denote significant effects of N for the occasion indicated: + = ( $P < 0.05$ ), ++ = ( $P < 0.01$ ) and +++ = ( $P < 0.001$ ). For further details see text.



The response of carboxylation efficiency ( $\epsilon$ ), to CO<sub>2</sub> and N treatments through time as days relative to anthesis, is shown for the various leaves of experiments One to Four in Figure 3.4, (a-f). Regardless of specific treatments, the same general pattern as found for leaf N content emerged, in which absolute values of  $\epsilon$  declined with time through leaf ontogeny, this decline being immediate from full emergence in non-flag leaves, but generally delayed in flag leaves prior to anthesis, particularly for leaves of higher N treatments (Fig 3.4, a, e and f). For all leaves and within a given measurement occasion, the effect of N treatments generally resulted in lower absolute values of  $\epsilon$  where lesser amounts of N had been applied during growth, these effects being highly significant ( $P < 0.001$ ) on all occasions for flag leaves, except for the second occasion of Experiment Two (Fig 3.4 c) where the effect was less significant ( $P < 0.05$ ), and the first occasion of Experiment One, where there was no significant effect (Fig 3.4 a). For flag-2 leaves of Experiment Three (Fig 3.4 d), there were no significant effects of N on  $\epsilon$ , and in flag-1 leaves, significant effects were only observed on the third occasion in Experiment Two (Fig 3.4 b;  $P < 0.01$ ) and first occasion in Experiment Three (Fig 3.4 d;  $P < 0.001$ ). Thus, in terms of the ability of different N treatments to exert effects on absolute values of  $\epsilon$  at a given time, the application of N treatments appears to have been particularly successful in flag leaves of Experiments One to Three. However, the relative magnitude of difference between values of  $\epsilon$  as exerted by N, were generally greater in experiments One and Three, than in Experiment Two, as observed above for leaf N content (Fig 3.3 a-e).

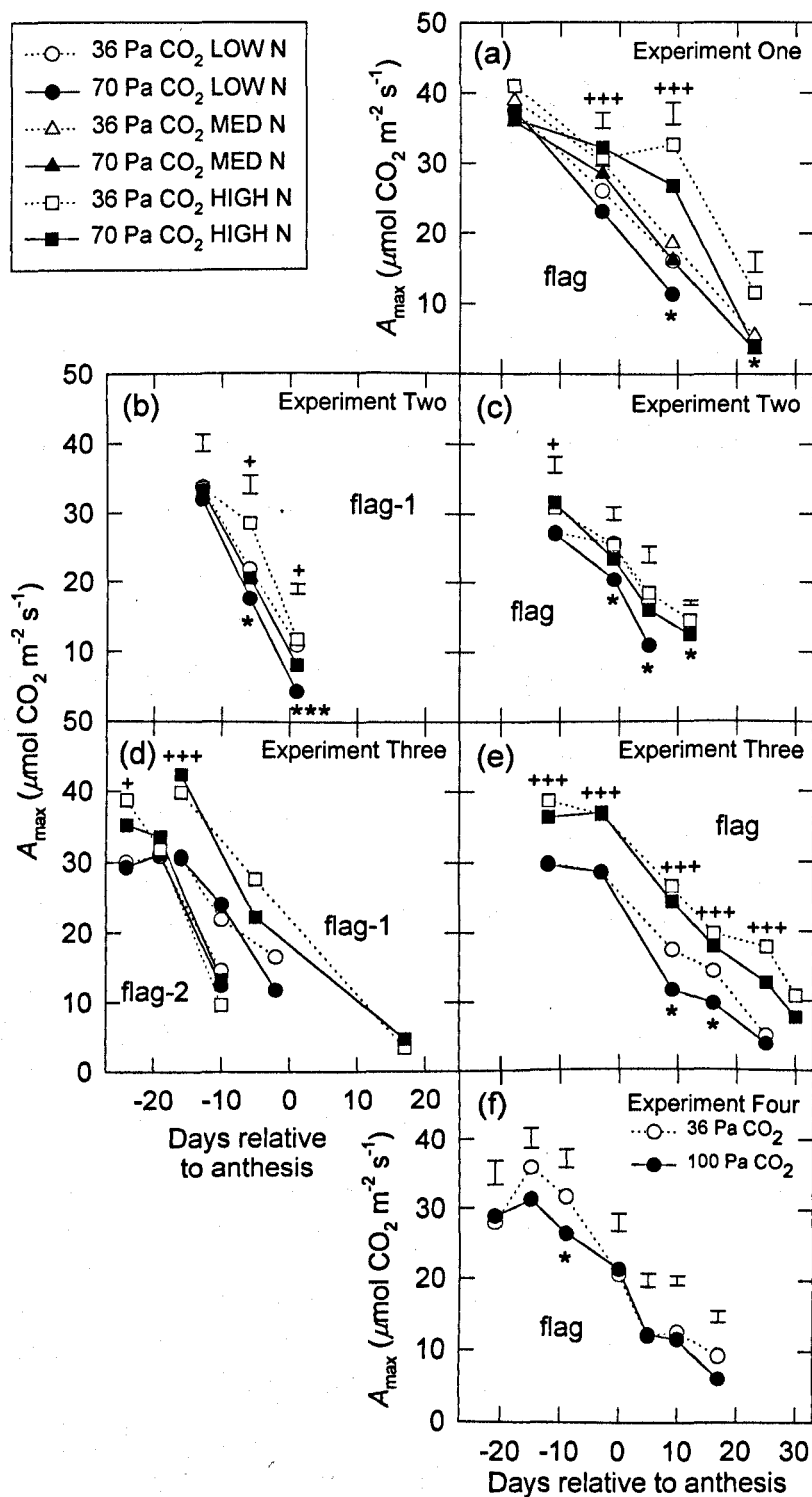
Since absolute values for  $\epsilon$  were generally lower or higher in the low and high N treatments respectively for a given measurement occasion, this meant that with

time and eventual leaf senescence,  $\varepsilon$  approached zero first in low, then medium and finally high N treated leaves, again following a similar pattern to that of leaf N content.

Superimposed upon these patterns of decline in  $\varepsilon$  due to different N treatments, were effects of CO<sub>2</sub> enrichment. However, CO<sub>2</sub> enrichment had no significant effect on  $\varepsilon$  in any leaf of any experiment on first measurement occasions corresponding to full leaf emergence (Fig 3.4 a-f), and indeed, no effect on any occasion in flag-2 leaves of Experiment Three (Fig 3.4 d). In flag-1 leaves of Experiment Two (Fig 3.4 b), highly significant effects ( $P<0.001$ ) of CO<sub>2</sub> enrichment on  $\varepsilon$  were observed at six days prior and one day after anthesis, decreasing  $\varepsilon$  by 41 and 46% respectively, in 70 compared to 36 Pa CO<sub>2</sub> grown leaves. In flag-1 leaves of Experiment Three, the only significant effect ( $P<0.05$ ) of CO<sub>2</sub> enrichment was at two days prior to anthesis for low N grown plants, where  $\varepsilon$  was decreased by 27% in 70 Pa CO<sub>2</sub> grown plants. With the exception of Experiment Four, no significant effect of CO<sub>2</sub> enrichment on  $\varepsilon$  was observed in flag leaves until anthesis, after which the effects persisted through grain filling and into senescence (Fig 3.4 a, c and e). For example, at 10 days after anthesis in flag leaves of Experiment One,  $\varepsilon$  was very significantly decreased ( $P<0.001$ ) by 32% in leaves grown at 70 compared to 36 Pa CO<sub>2</sub>; significantly ( $P<0.001$ ) by 20% at anthesis in flag leaves of Experiment Two, and significantly ( $P<0.05$ ) by 47% at nine days after anthesis in flag leaves of Experiment Three. For flag leaves of Experiment Four, the only significant effect of CO<sub>2</sub> enrichment ( $P<0.05$ ) was observed at nine days prior to anthesis but still 12 days subsequent to full emergence, when  $\varepsilon$  was decreased by 30% in flag leaves grown at 100 compared to 36 Pa CO<sub>2</sub>. Where significant effects of CO<sub>2</sub> enrichment

were exerted and observed, they were generally greater on leaves of low compared to high N grown plants (e.g. Fig 3.4 e), but the only significant ( $P < 0.05$ ) interaction between CO<sub>2</sub> and N on  $\epsilon$  was observed in flag leaves of Experiment Three, between 12 days prior and 16 days after anthesis (Fig. 3.4 e). As values for  $\epsilon$  were almost always lower (even when non-significant) in 70 compared to 36 Pa CO<sub>2</sub> grown leaves for a given N treatment within a given occasion, this meant that  $\epsilon$  declined fastest in CO<sub>2</sub> enriched leaves.

The response of  $A_{\max}$ , derived from the asymptote of  $A/pC_i$  responses, and indicative of the activity of components determining RuBP-regeneration capacity, or, possibly stromal PO<sub>4</sub><sup>3-</sup> regeneration capacity, to CO<sub>2</sub> and N treatments through time as days relative to anthesis, is shown below for the various leaves of experiments One to Four (Fig. 3.5 a-f). As for leaf N content and  $\epsilon$ , regardless of specific treatments, a general pattern emerged in which  $A_{\max}$  values declined with time through leaf ontogeny, this decline being immediate from full emergence in non-flag leaves, but sometimes slightly delayed in flag leaves (Fig. 3.5 e & f). For all leaves of all experiments, the pattern of significant effects of N treatments on  $A_{\max}$ , very closely mirrored those of N on  $\epsilon$ , being identical in flag leaves of Experiment One and in flag-1 and flag leaves of Experiment Three. In flag-2 leaves of Experiment Three, the first and only observed effect of any treatment on any parameter measured for this leaf in this study, occurred at full emergence, where  $A_{\max}$  was significantly ( $P < 0.05$ ) lower in low N grown leaves.



**Figure 3.5** The maximum rate of net photosynthesis under saturating light and  $\text{CO}_2$  ( $A_{\max}$ ) versus time as days relative to anthesis, in flag-2 (d), flag-1 (b & d) and flag leaves (a, c, e & f) of spring wheat grown in four separate experiments, at either 36 or 70 Pa  $\text{CO}_2$  (Experiments One to Three, panels a-e), and 36 or 100 Pa  $\text{CO}_2$  (Experiment Four, panel f). Nitrogen applications during growth are indicated in the key. Bars are 1 x SED from  $\text{CO}_2 \times \text{N}$  ANOVA, except in Experiment Four, where bar is SED from  $\text{CO}_2 \times \text{Time}$  ANOVA, and for all,  $n = 6$ . Asterisks denote significant effects of  $\text{CO}_2$  for the occasion indicated: \* = ( $P < 0.05$ ), \*\* = ( $P < 0.01$ ) and \*\*\* = ( $P < 0.001$ ). Plus signs denote significant effects of N for the occasion indicated: + = ( $P < 0.05$ ), ++ = ( $P < 0.01$ ) and +++ = ( $P < 0.001$ ). For further details see text.

For flag leaves of Experiment Two, a significant effect ( $P < 0.05$ ) was only observed at 11 days prior to anthesis. In all instances where effects were observed, absolute values of  $A_{\max}$  were always lower in leaves grown with least N, so that  $A_{\max}$  approached zero first in low, then medium and finally high N treated leaves with time. For flag leaves of Experiment Two different N treatments had less effect on mean absolute values of  $A_{\max}$  than N treatments had done on mean absolute values of  $\varepsilon$  for the same leaves (Fig. 3.4 c compared with Fig. 3.5 c). This was not the case for flag leaves of Experiments One and Three, where N treatments exerted similar effects on mean absolute values of both  $\varepsilon$  and  $A_{\max}$  (Fig. 3.4 a & e compared with Fig. 3.5 a & e).

Carbon dioxide enrichment had no significant effect on  $A_{\max}$  in any leaf of any experiment on the first measurement occasion corresponding to full leaf emergence, and with the exception of flag leaves from Experiment Four, and second occasion of flag-1 leaves of Experiment Two, on no occasion between this time and anthesis. Thereafter, and in leaves before flag, the only significant effects of  $\text{CO}_2$  enrichment were observed at one day after anthesis ( $P < 0.001$ ) for flag-1 leaves of Experiment Two, where  $A_{\max}$  was decreased by 45% in leaves of 70 compared to 36 Pa  $\text{CO}_2$  grown plants. In flag leaves of Experiment Four,  $A_{\max}$  was significantly decreased ( $P < 0.05$ ) by 17% in leaves of 70 compared to 36 Pa  $\text{CO}_2$  grown plants at nine days before anthesis, but nevertheless, still 12 days from measurement occasion one. In all other flag leaves, there was no significant effect of  $\text{CO}_2$  enrichment on  $A_{\max}$ , until anthesis (Experiment Two) or well into the grain filling period at least seven days after anthesis (Experiments One and Two), from where the effects persisted through the remainder of ontogeny. There was no significant interaction

between CO<sub>2</sub> and N on  $A_{\max}$  in any leaf of any experiment. There was therefore no evidence of the slight stimulation of  $A_{\max}$  at elevated CO<sub>2</sub> found by Delgado et al., (1994).

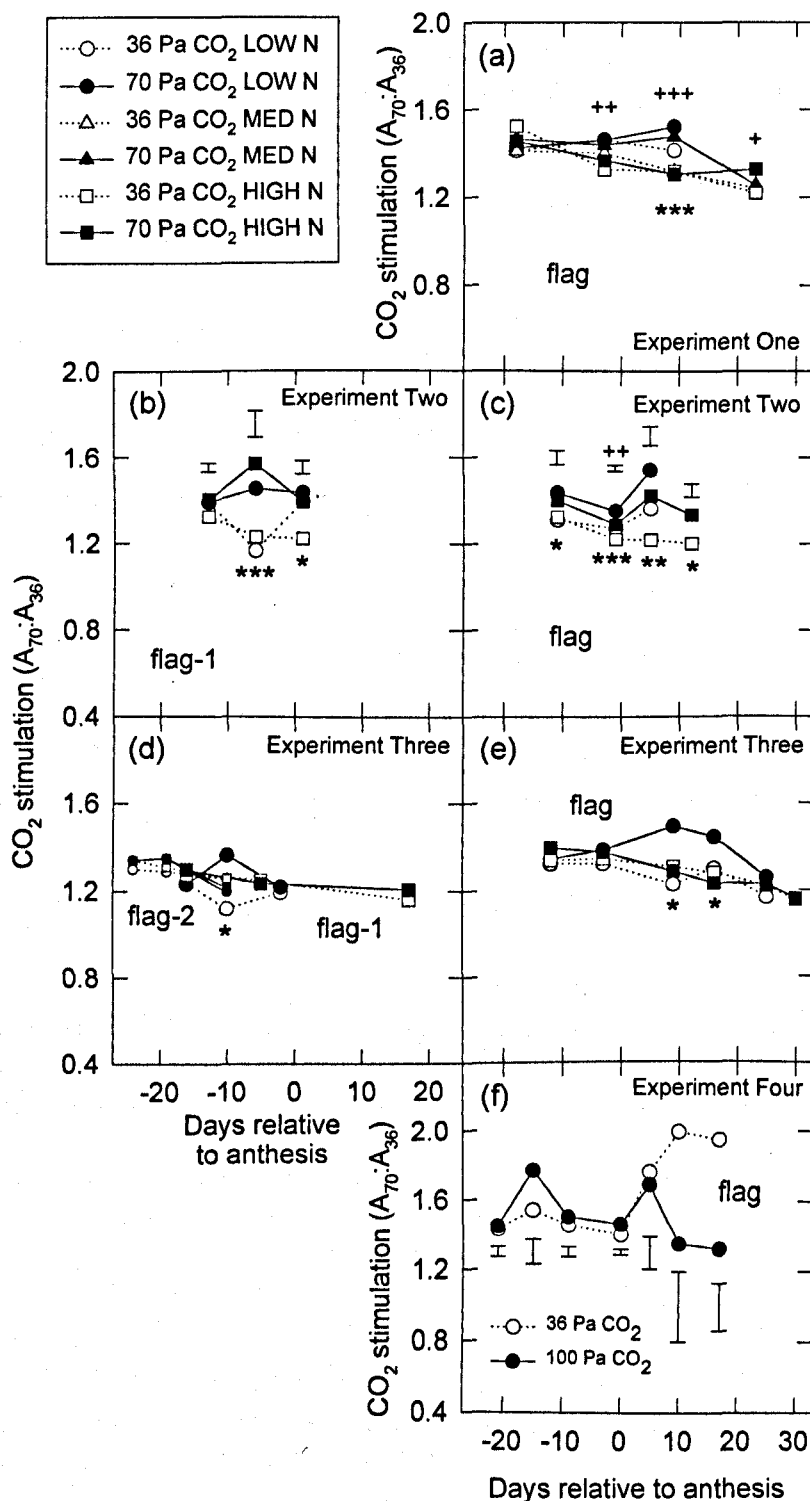
Importantly, the effects of CO<sub>2</sub> enrichment on  $A_{\max}$  were never generally as highly significant as the effects of CO<sub>2</sub> enrichment on  $\varepsilon$  for leaves of experiments One and Two, or as great in magnitude. For example, at 10 days after anthesis for flag leaves of Experiment One, in 70 compared to 36 Pa CO<sub>2</sub> grown plants, there was a 32% greater decline in  $\varepsilon$  (Fig. 3.4 a), but only a 20% decline in  $A_{\max}$  (Fig. 3.5 a). In flag leaves of Experiment Three a similar pattern was seen from nine days after anthesis, where on this occasion, a 47% greater decline in  $\varepsilon$  (Fig. 3.4, e), but again smaller 33% decline in  $A_{\max}$  (Fig. 3.5 e), was observed, as were very similar patterns in other leaves of other experiments. Since  $\varepsilon$  and  $A_{\max}$  respectively are measures of the *in vivo* capacity to carboxylate and regenerate RuBP, this result suggests that there might have been some rebalancing of *in vivo* capacities in plants grown at 70 Pa CO<sub>2</sub>, with a relative ratio change in favour of RuBP-regeneration, since  $\varepsilon$  appeared to decline proportionately faster.

To test this further, the CO<sub>2</sub> stimulation effect was determined for individual  $A/pC_i$  responses, where CO<sub>2</sub> stimulation is calculated from the ratio of photosynthetic rates obtained at  $pC_a = 36$  ( $A_{36}$ ) and 70 ( $A_{70}$ ) Pa CO<sub>2</sub>. For plants grown long-term at 36 Pa CO<sub>2</sub> the CO<sub>2</sub> stimulation is typically 1.4 (Farquhar and von Caemmerer, 1981). However, for plants grown long-term at 70 Pa CO<sub>2</sub> and if there is a rebalancing of capacities with a relative increase in RuBP-regeneration over carboxylation such that both co-limit at  $pC_a=70$  Pa, then theory predicts that the ratio could increase to 1.8 (Sage, 1994). The ratio of  $A_{70}:A_{36}$  is a direct empirical

measure of whether capacities have been altered to increase efficiency at the growth  $pC_a$ ; in which case a greater ratio would be expected at elevated  $pC_a$  (Sage, 1994).

For all leaves of all experiments, N treatments had no significant effect on CO<sub>2</sub> stimulation at full emergence, or on any subsequent occasion in leaves of Experiments Three and Four (Fig. 3.6 d, e & f). There were however significant effects in flag leaves of Experiment One on all occasions subsequent to emergence (Fig 3.6 a;  $P<0.01$ ,  $P<0.001$  and  $P<0.05$ ), in flag-1 leaves of Experiment Two on occasion three (Fig 3.6 b;  $P<0.05$ ) and flag leaves of Experiment Two on occasions two and three (Fig. 3.6 c;  $P<0.01$  and  $P<0.05$  respectively). In all these instances, CO<sub>2</sub> stimulation was increased in leaves of the lower N grown plants.

With the exception of flag leaves of Experiment Two, there was no significant effect of CO<sub>2</sub> enrichment on CO<sub>2</sub> stimulation in any leaf of any other experiment on the first measurement occasion corresponding to full leaf emergence, nor on any subsequent occasion for flag-2 leaves of Experiment Three (Fig. 3.6 d) or flag leaves of Experiment Four (Fig. 3.6 f). In flag-1 leaves of Experiment Two (Fig 3.6 b) significant effects developed midway through ontogeny, being highly significant ( $P<0.001$ ) on occasion two and significant on occasion three ( $P<0.05$ ), where CO<sub>2</sub> stimulation was increased by 26 and 8% respectively, in leaves of 70 compared to 36 Pa CO<sub>2</sub> grown plants. For flag-1 leaves of Experiment Three, there was a significant ( $P<0.05$ ) effect of increased CO<sub>2</sub> stimulation in 70 Pa CO<sub>2</sub>, low N grown leaves at 10 days prior to anthesis. For remaining flag leaves, in Experiment One, CO<sub>2</sub> enrichment had a highly significant ( $P<0.001$ ) effect 10 days after anthesis, with the greatest CO<sub>2</sub> stimulation being on the lowest N treatment grown



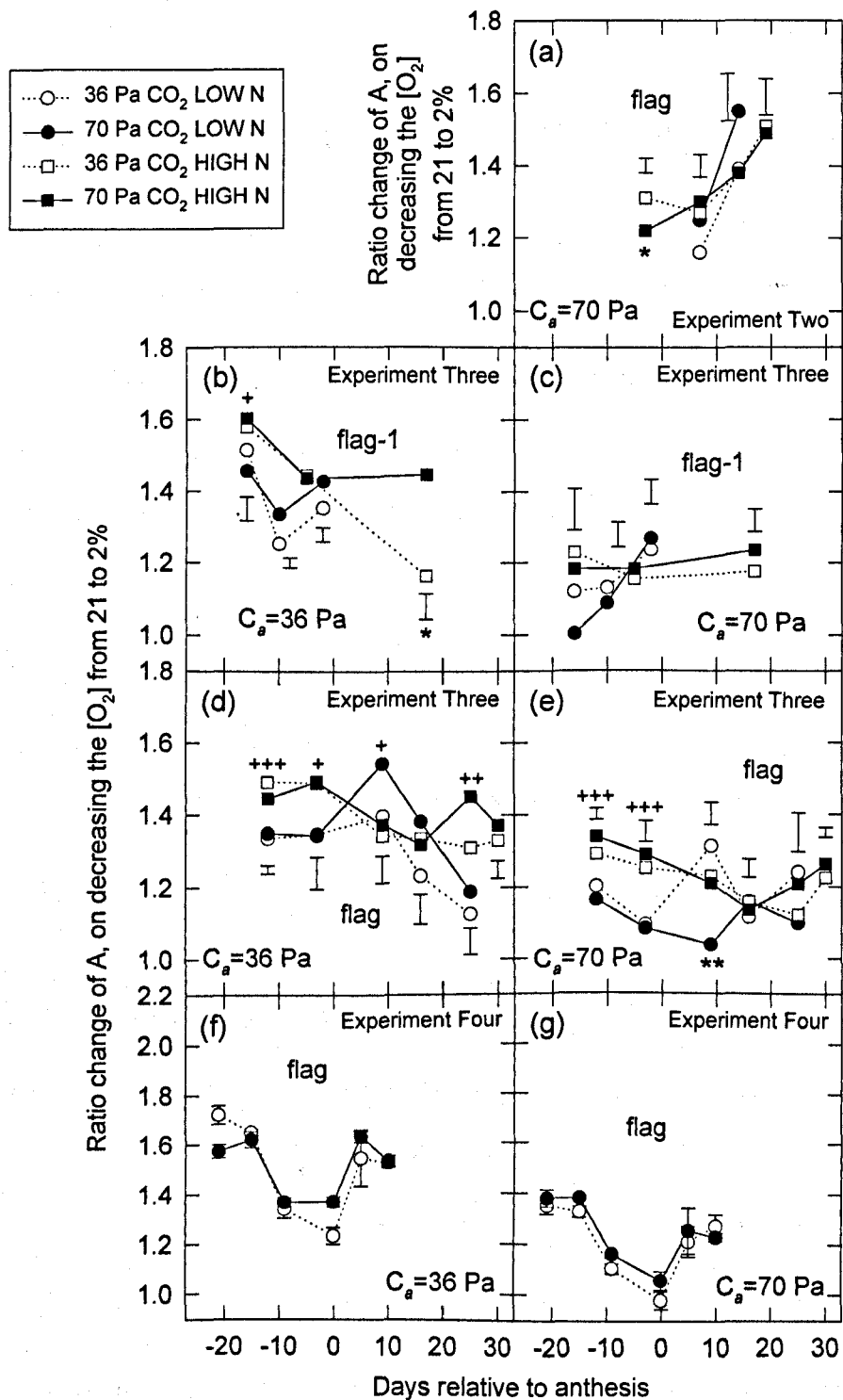
**Figure 3.6** CO<sub>2</sub> stimulation ( $A_{70}:A_{36}$ ) versus time as days relative to anthesis, in flag-1 (b & d) and flag leaves (a, c, e & f) of spring wheat grown in four separate experiments, at either 36 or 70 Pa CO<sub>2</sub> (Experiments One to Three, panels a-e), and 36 or 100 Pa CO<sub>2</sub> (Experiment Four, panel f). Nitrogen applications during growth are indicated in the key. Bars are 1 x SED from CO<sub>2</sub> x N ANOVA, and for all, n = 6. Asterisks denote significant effects of CO<sub>2</sub> for the occasion indicated: \* = ( $P < 0.05$ ), \*\* = ( $P < 0.01$ ) and \*\*\* = ( $P < 0.001$ ). Plus signs denote significant effects of N for the occasion indicated: + = ( $P < 0.05$ ), ++ = ( $P < 0.01$ ) and +++ = ( $P < 0.001$ ). For clarity, slightly smaller symbols have been used for flag-2 leaves (panel d). For further details see text.



at elevated CO<sub>2</sub>. In flag leaves of Experiment Three, significant ( $P<0.05$ ) CO<sub>2</sub> effects on stimulation were observed from 9 to 16 d after anthesis, and again, lower N grown treatments were stimulated more (Fig. 3.6 e).

For flag leaves of Experiment Two (Fig. 3.6 c), significant effects of CO<sub>2</sub> enrichment on CO<sub>2</sub> stimulation were observed on all measurement occasions, although effects were more highly significant on occasions two ( $P<0.001$ ) and three ( $P<0.01$ ), corresponding to occasions at anthesis and five days after. The only significant interaction between N and CO<sub>2</sub> enrichment on CO<sub>2</sub> stimulation was observed at 9 days after anthesis in flag leaves of Experiment One, and between 9 and 16 days after anthesis, in the flag leaves of Experiment Three (Fig 3.6 e).

To determine whether any changes to the *in vivo* balance between capacities for RuBP-regeneration and PO<sub>4</sub><sup>3-</sup>-regeneration were occurring, the O<sub>2</sub> sensitivity response of photosynthesis at various  $pC_a$  was determined. Regardless of specific treatments, the stimulation of photosynthesis on decreasing the O<sub>2</sub> concentration from 20 to 2 kPa was greatest (up to 72%) when measured at  $pC_a=36$  (Fig 3.7 b, d & f), and still highly stimulated (maximum 54%) when measured at  $pC_a=70$  (Fig 3.7 a, c, e & g). Nevertheless, the magnitudes of stimulation were comparable in range to those reported elsewhere for O<sub>2</sub> sensitivity responses (Sage and Sharkey, 1987; Paul et al., 1990; Makino, 1994). With time, there was no consistent pattern in general trends across experiments, for the stimulation of photosynthesis on decreasing the O<sub>2</sub> concentration. In flag leaves of Experiment Two for example, the response generally increased with time, in flag-1 leaves of Experiment Three was reasonably constant, whilst in flag leaves of Experiment Three, the percentage of



**Figure 3.7** Ratio of the change in photosynthetic rate on decreasing the  $[O_2]$  from 21 to 2kPa, measured at  $pC_a = 20$  (f), 36 (b, d & g) or 70 (a, c, e & h) Pa, against days relative to anthesis, in flag-1 (b & d) and flag leaves (a, d, e, g, g & h), from Experiments Two (a), Three (b, c, d & e) and Four (f, g & h). Bars are 1 x SED from  $CO_2 \times N$  ANOVA in experiments Two and Three, and 1 x Std Err in Experiment Four. For all,  $n = 6$ . Asterisks denote significant effects of  $CO_2$  for the occasion indicated: \* = ( $P < 0.05$ ) and \*\* = ( $P < 0.01$ ), and plus signs denote significant effects of N for the occasion indicated: + = ( $P < 0.05$ ), ++ = ( $P < 0.01$ ) and +++ = ( $P < 0.001$ ). For further details see text.

photosynthetic stimulation tended to decrease. In flag leaves of Experiment Four the stimulation response declined between full emergence and anthesis, and then showed a subsequent increase.

Nitrogen treatments only had limited effects on O<sub>2</sub> sensitivity responses. In flag-1 leaves of Experiment Three measured at  $pC_a = 36$  Pa (Fig. 3.7 b), N had a significant effect ( $P < 0.05$ ) on the first occasion only, causing a 7% increase in the stimulation response, in high compared to low N grown leaves.

However, this significant effect was lost, when the stimulation of photosynthesis was measured at  $pC_a = 70$  Pa CO<sub>2</sub> (Fig 3.7 c). Similarly, significant effects of N on the stimulation of photosynthesis on decreasing the percentage O<sub>2</sub> were observed in flag leaves of Experiment Three on the first three and fifth occasions when measured at  $pC_a = 36$  Pa, but only on the first two occasions when the same leaves were measured at  $pC_a = 70$  Pa.

Similarly, effects of CO<sub>2</sub> enrichment on the O<sub>2</sub> sensitivity response were also very limited, with effects only occurring on one occasion per leaf in those few leaves where any effect was observed. In flag leaves of Experiment Two for example, a significant effect ( $P < 0.05$ ) was seen on the first occasion of measurement, where the sensitivity response was 6% less in high N leaves grown at 70 Pa CO<sub>2</sub>. Likewise, on the third occasion for flag leaves of Experiment Three measured at  $pC_a = 70$  Pa (Fig. 3.7 e) the O<sub>2</sub> sensitivity response was significantly ( $P < 0.01$ ) 21% less in low N leaves grown at 70 Pa CO<sub>2</sub>, although this effect had not been observed for the same leaves when tested at  $pC_a = 36$  Pa (Fig. 3.7 d). The only other significant effect ( $P < 0.05$ ) of CO<sub>2</sub> enrichment on O<sub>2</sub> sensitivity was observed on the third occasion for flag-1 leaves of Experiment Three measured at  $pC_a = 36$  Pa, where in contrast to previous

responses, the O<sub>2</sub> sensitivity response was decreased by 20% in 36 Pa CO<sub>2</sub> grown leaves. The only significant interaction between CO<sub>2</sub> and N on the O<sub>2</sub> sensitivity response, occurred in flag leaves of Experiment Three, nine days after anthesis and when  $pC_a = 70$  Pa (Fig. 3.7 e).

Finally, zero O<sub>2</sub> sensitivity was only observed on two occasions, for low N and 70 Pa CO<sub>2</sub> grown flag-1 leaves of experiment three on the first occasion measured at  $pC_a = 70$  Pa (Fig. 3.7 c), and for low N and 36 Pa CO<sub>2</sub> grown flag leaves of experiment Four on occasion four (anthesis), also measured at  $pC_a = 70$  Pa (Fig. 3.7 g). Apart from these two occasions, leaves always demonstrated O<sub>2</sub> sensitivity, with a stimulation response ranging from 5 to 90%, implying that PO<sub>4</sub><sup>3-</sup> regeneration capacity was never completely limiting at 70 Pa CO<sub>2</sub>.

### *Discussion*

The general decline in leaf N content with time and regardless of specific treatments, has been reported extensively elsewhere, and reflects a natural ontogenic decline of components and resources within leaves as they are re-mobilised to sinks. For most non-flag leaves these sinks will be younger leaves developing higher in the canopy, and for penultimate and flag leaves, the predominant sink will be ears and developing grains within them. Given this re-mobilisation of N, it is of little surprise that leaves which contain lesser amounts of N to begin with, senesce earlier because this resource is even less abundant, as is clearly seen in the data presented here, and has been reported elsewhere (Evans, 1983; Makino et al., 1983; Lawlor et al., 1989). As a result, this background ontogenic decline has to be considered at all times, as any other effects observed will be superimposed upon it.

That the absolute differences between leaf N contents of various N treatments was far greater in flag leaves of Experiments One and Three (Fig. 3.3 a & d), than in flag leaves from Experiment Two (Fig 3.3 c), is likely to be a reflection of the manner and timing of N applications, as reference to Figure 2.1 will help explain. In Experiment One (Fig. 2.1 a), targets for the total amount of N to apply for three different treatments were determined at the start of the experiment, so that from the first application, all three treatments received a weekly application but of different amounts. Applications ceased at 25 days before anthesis for both the low and medium N treatments, but an additional and final application was made for the high N treatment at 8 days prior to anthesis. Conversely, in Experiments Two and Three, again, final targets were determined at the start of experiments, but weekly applications contained the same amount of N, and once targets were reached, further supply of exogenous N ceased. Therefore, subsequent plant development was dependant upon N and other minerals already in the plant. In Experiment Two, plants of both low and high N treatments received final N applications 30 days prior to anthesis, and in Experiment Three at 37 and 7 days before anthesis for low and high N treatments respectively.

Thus, not only was maximum flag leaf area attained, but additional N was available for further investment into the photosynthetic machinery, and hence the observed differences in absolute N contents per unit leaf area between N treatments and experiments. Indeed, in summary it can be said that for low and medium N treatments and both treatments of Experiment Two, N applications exerted more of an effect on leaf area, whilst for high N treatments of experiments One and Three, N applications exerted more of an effect on leaf N content per unit area.

Leaves of plants grown with limiting N supplies achieve senescence earlier, and CO<sub>2</sub> enrichment positively enhances this further. In order to offer an explanation for this, it is first necessary to consider N re-mobilisation and the grain filling process in more detail. The latter can be sub-divided into three phases (Waters et al., 1980); (1) an initial lag phase lasting for approximately five days from anthesis during which rapid cellular division takes place in the developing grains and limited re-mobilisation occurs, (2) a rapid phase of growth between 6 and 25 days after anthesis when these cells grow and take up the bulk of re-mobilised N, and (3) the end of the rapid phase of growth.

For wheat, Simpson et al., (1983) have shown that to the grain, leaves contribute 40% of N (of which flag leaves contribute 25% or 10% of the total), glumes 23%, stems 23% and roots 16%, and that over 80% of this N is supplied as either glutamate, aspartate, serine, alanine or glycine. Interestingly, Simpson et al., (1983) also demonstrated that for the contribution of amino acids from leaves to grains, less than 50% went directly, with at least 54% going via the phloem to the roots.

From the biomass data reported in Chapter Two, it is apparent that at anthesis, 70 Pa CO<sub>2</sub> grown plants had greater ear dry mass than those at 36 Pa CO<sub>2</sub>, and that at final harvest, individual ears of 70 Pa CO<sub>2</sub> grown plants contained more grains, with similar %N per grain and individual grain mass compared to grain from 36 Pa CO<sub>2</sub> grown plants. Therefore, through the grain filling period it would seem that ears and grains of 70 Pa CO<sub>2</sub> grown plants must have had greater sink strength, both for N and C. The extra demand for C could be met by increased photosynthesis at elevated  $pC_a$ ; the extra demand for N could only be met by

relocating more N to the grain from other parts of the plant, including from flag leaves.

Additional evidence to support the above as an explanation, may be derived from the fact that in general, significant effects of CO<sub>2</sub> on the accelerated loss of N from leaves of plants grown at 70 Pa CO<sub>2</sub>, were not seen until seven to eight days after anthesis, corresponding with the start of the rapid period of grain filling (Simpson et al., 1983), suggesting a tight linkage between this treatment and the grain filling event.

In one study on source-sink relations in wheat (Koide and Ishiara, 1992), the effect of entire ear removal at seven days after anthesis on photosynthesis and flag leaf biochemistry through to the end of grain fill was studied. Through this period, there were no differences between control and no-ear plants in apparent quantum yield, or chlorophyll content. However, in control plants (ears intact) from 16 days after anthesis (corresponding to the middle of the rapid phase of grain fill) there was a more rapid decline in photosynthesis and carboxylation efficiency, as a result of a faster decline in Rubisco and other soluble proteins. Interestingly, Rubisco decreased proportionately faster than chlorophyll in control plants, but more in parallel in the no-ear treatments. In a study on rice in which panicles were removed at anthesis (Nakano et al., 1995), again no effects were seen prior to 10 to 15 days after ear removal and anthesis, but then corresponding with the maximum grain fill period, photosynthesis and amounts of leaf N, Rubisco, cytochrome *f*, SPS, AGPase and unlike the previous study, chlorophyll, all declined more rapidly in flag leaves of control plants. Thus, taken together, the results of these two examples, despite representing extremes of sink size in ears suggest that it is the sink size of

ears/panicles, and therefore the number of developing grains within them, which determines the rate at which N is re-mobilised from leaves. This in turn lends further support to the explanation for the CO<sub>2</sub> enhancement effect on leaf senescence via increased N re-mobilisation reported here.

In addition to bigger ears at elevated CO<sub>2</sub> acting as stronger sinks for resources, some authors have reported that senescence and the loss of N from leaves may be enhanced due to overall faster crop development at 70 Pa CO<sub>2</sub>. For example in Pinter et al., (1995), parallel studies of a wheat crop through development under FACE at 55 Pa CO<sub>2</sub>, indicated that anthesis occurred one to two days earlier and grain filling was complete seven days earlier in 55 compared to 36 Pa CO<sub>2</sub> grown plants. Plants grown at 55 Pa CO<sub>2</sub> showed an average increase in day time temperature of 0.56°C relative to controls, which was associated with an 11% decrease in evapotranspiration, probably due to partial stomatal closure caused by the elevated CO<sub>2</sub> atmosphere (Kimball et al., 1986). Since a major factor controlling crop development is thermal time (Rawson and Zajac, 1993), the cumulative effect of a 0.56°C increase would substantially increase the accumulation of degree days, therefore enhancing phenological development and bringing forward the onset of senescence. However, from the results presented in Chapter Two for various stages of development in Experiments One and Three of this study, there was no evidence for a CO<sub>2</sub> effect on the rate of phenological development. Indeed, for the low N treatment of both experiments, 50% anthesis was approximately one to two days later at 70 compared to 36 Pa CO<sub>2</sub> (Table 2.4). This suggests that for the experiments reported in this study, the accelerated loss of N from leaves grown at 70 Pa CO<sub>2</sub> was most likely entirely due to increased sink strength from larger ears with



more grain.

It has been extensively reported that the photosynthetic capacity of a leaf is closely related to leaf N content (von Caemmerer and Farquhar, 1981; Evans, 1983; Evans and Terashima, 1988; Sage et al., 1990; Makino et al., 1992). Since the parameters of carboxylation efficiency and  $A_{\max}$  are derived from the  $A/pC_i$  responses of leaves, it is not unexpected that the patterns of decline that these parameters show for plants of different N and CO<sub>2</sub> treatments, in general, closely mirror declines in leaf N content. Excluding Experiment Four, CO<sub>2</sub> enrichment consistently had no effect on N content,  $\epsilon$  or  $A_{\max}$  in flag leaves, until or shortly after anthesis. Thus growth of plants at 70 Pa CO<sub>2</sub> clearly accelerated the loss of N and decline in values for  $\epsilon$  and  $A_{\max}$ . This is well illustrated in the pattern of decline in flag leaves of Experiment Three, with the greatest effect of CO<sub>2</sub> on all three of these parameters occurring not before nine days after anthesis, and always exerting the strongest effect on low N treated leaves. Therefore, the same arguments given above for the effects of CO<sub>2</sub> and N application on leaf N content apply for the general patterns of decline in  $\epsilon$  and  $A_{\max}$  through time.

Thus, from an analysis of the effects of CO<sub>2</sub> enrichment on values of  $\epsilon$  or  $A_{\max}$  in various leaves through ontogeny of four separate experiments, a general pattern begins to emerge in which significant CO<sub>2</sub> effects are never seen at full leaf emergence, but develop (with the exception of Experiment Four) at or subsequent to anthesis and on through the period of grain filling to senescence, resulting in a faster loss of the parameter being measured at the higher growth CO<sub>2</sub> concentration. However, the occurrence of effects and degree to which they are significant, were generally far more frequent and higher for  $\epsilon$  than  $A_{\max}$ . As  $\epsilon$  is a reflection of the

total amount of catalytically competent Rubisco active sites in leaves (Sage et al., 1989) and this in turn determines the carboxylation capacity, and  $A_{\max}$  reflects the amount and activity of components determining RuBP-regeneration capacity, a faster relative decline in  $\varepsilon$  compared to  $A_{\max}$  at 70 Pa CO<sub>2</sub> may reflect a preferential decline in carboxylation relative to RuBP-regeneration capacity which would represent a re-balancing of capacities to some degree. Therefore, to determine whether or not a re-balancing was taking place, the ratio of  $A_{70}:A_{36}$  (CO<sub>2</sub> stimulation) was determined for all leaves of all four experiments (Fig 3.6 a-f), since if  $\varepsilon$  does decline proportionately faster than  $A_{\max}$ , this will change the shape of the  $A/pC_i$  curve, increasing  $A_{70}:A_{36}$ .

For the CO<sub>2</sub> stimulation ratio, a general pattern emerged (Fig. 3.6) in which at full expansion no significant effect of growth at elevated CO<sub>2</sub> was observed in any leaf of any experiment, so that a ratio of 1.4 remained fairly constant, indicating that no change had occurred in the *in vivo* balance between carboxylation and RuBP-regeneration capacities. Indeed, this pattern and ratio was maintained for all subsequent occasions of flag-2 leaves in Experiment Three, this representing the lowest canopy leaf measured in any experiment of this study. However, for leaves of higher insertion in the canopy and between 10 days prior and during anthesis for flag-1 leaves of Experiments Two and Three (Fig. 3.6 b & d), and between anthesis and 16 days after in flag leaves of Experiments One to Three (Fig. 3.6 a, c & e), significant effects of growth at 70 Pa CO<sub>2</sub> were observed resulting in increased ratios and strongest effects in the most N limited plants. Thus the timing of these responses generally coincided with grain filling, when the demand for N was high and particularly so in low N 70 Pa CO<sub>2</sub> grown plants with more grains per ear (Table

2.9) than 36 Pa CO<sub>2</sub> grown controls. These results strongly suggest that there was a CO<sub>2</sub> induced change in the *in vivo* balance of capacities, with a preferential decrease in the carboxylation capacity such that this response was in the direction as suggested by the hypothesis being tested.

However, it should be noted that these responses were occurring late in the development of the leaves, since for example in the flag leaves of Experiment One at 10 days after anthesis, 77 and 68% respectively of  $\epsilon$  and  $A_{\max}$  measured at full leaf expansion had already been lost. Further, for a maximum re-balancing between carboxylation and RuBP-regeneration capacities at 70 Pa CO<sub>2</sub> an increase in the CO<sub>2</sub> stimulation ratio to 1.8 has been proposed, yet observed ratios never exceeded 1.6. Thus the late timing of the response during re-mobilisation of resources from leaves brings into question the benefit of this response to the plant, and further, the lower than predicted ratio increase suggests that only a partial re-balancing took place.

The average  $A_{70}:A_{36}$  stimulation of approximately 1.4 (Fig. 3.6) is compatible with the assumption that carboxylation and RuBP-regeneration limit photosynthesis at 36 and 70 Pa, respectively. If PO<sub>4</sub><sup>3-</sup>-regeneration limited photosynthesis at 70 Pa, a smaller stimulation would be expected; however this was further investigated with the O<sub>2</sub> sensitivity response of photosynthesis measured at  $A_{70}$ . It is clear, with the exception of two occasions in different experiments, that plants always showed a response, indicating that the capacity to export triose phosphate from chloroplasts and subsequently regenerate PO<sub>4</sub><sup>3-</sup> was never completely limiting. This suggests that there was still potential for improving the *in vivo* balance between carboxylation and RuBP-regeneration capacities further at 70 Pa CO<sub>2</sub>. It is perhaps also worth noting, that these responses were measured after the plants had already been exposed to

saturating irradiance for 2.5 h. If tested under growth conditions where irradiance was lower, even greater O<sub>2</sub> sensitivity may have been observed.

That there was no O<sub>2</sub> sensitivity response and only a 5% stimulation in 36 and 100 Pa CO<sub>2</sub> grown plants respectively of Experiment Four at anthesis is interesting, since shortly before anthesis, McKee and Woodward (1994) also observed a lack of O<sub>2</sub> sensitivity in 40 and 70 Pa CO<sub>2</sub> grown flag leaves of wheat measured at  $pC_a = 70$  Pa. However, these authors only measured this parameter on one occasion, whereas from the data reported here it is clear that following the decline in O<sub>2</sub> sensitivity prior to anthesis, a recovery was observed. This could reasonably be explained by a high photosynthetic capacity of newly emerged flag leaves prior to anthesis resulting in an accumulation of triose phosphate and subsequent limitation to PO<sub>4</sub><sup>3-</sup>-regeneration capacity, being alleviated after anthesis, not only because of a strong demand from developing grains for triose phosphate, but because of a slow and gradual decline in photosynthetic capacity resulting in decreased daily production. Further, that this pattern was not so apparent in leaves of other experiments could be explained by the fact that plants in Experiment Four were grown with the least amount of N compared to other experiments. Thus the demonstration in this study that the O<sub>2</sub> sensitivity response can vary considerably with time, warns against making conclusions about the response of various plant parameters to elevated CO<sub>2</sub> when only infrequent measurements have been taken.

It is highly probable that the significant effect of CO<sub>2</sub> on O<sub>2</sub> sensitivity at 9 days after anthesis in flag leaves of Experiment Three was entirely due to a very high stimulation in ambient low N grown plants above that of even high N grown plants, suggesting that this result is erroneous. If so, then with the exception of a significant

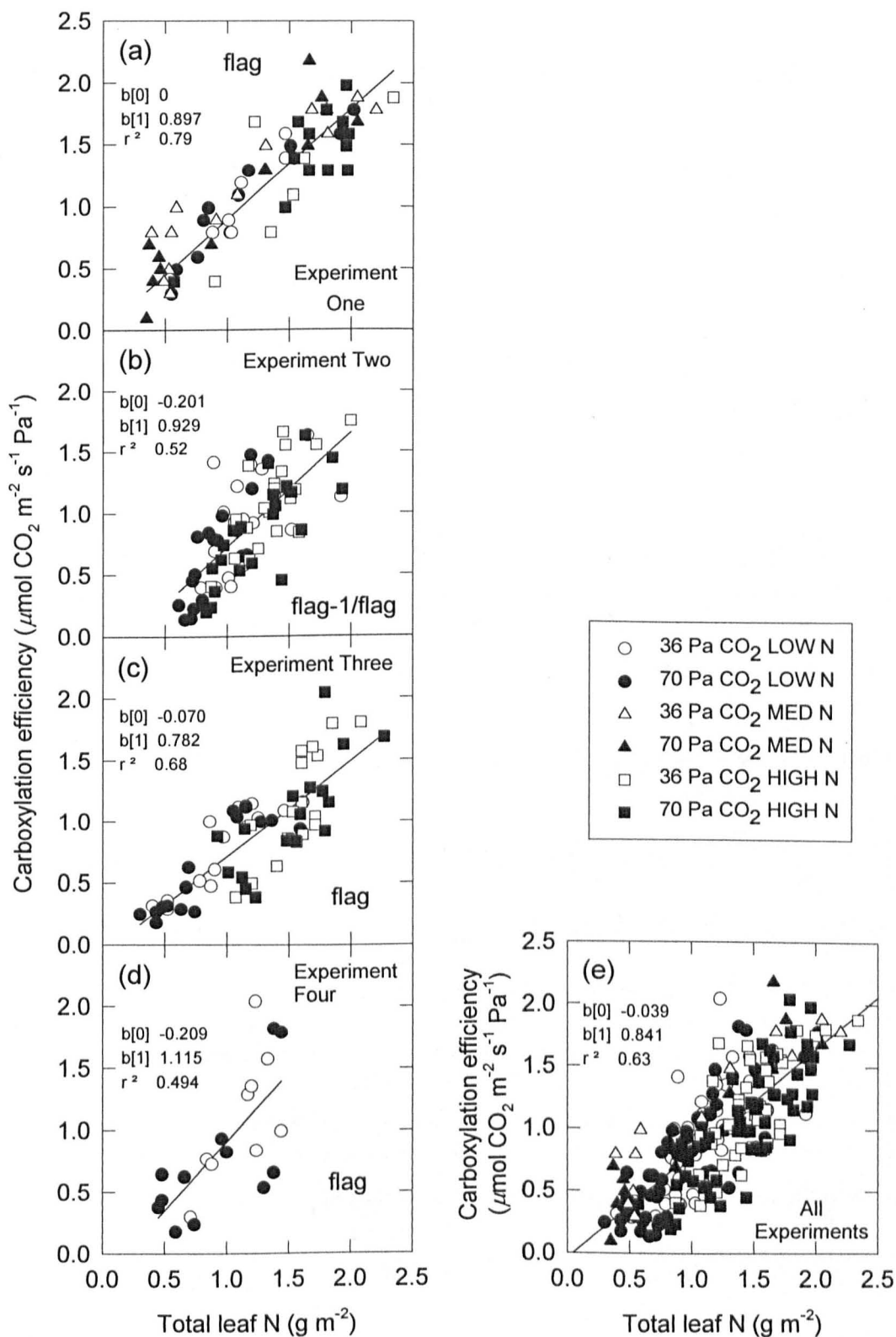
CO<sub>2</sub> effect in the 70 Pa CO<sub>2</sub>, high N grown leaves at 9 days before anthesis in Experiment One, significant CO<sub>2</sub> effects on the sensitivity of photosynthesis to decreased O<sub>2</sub> concentration are rare and inconsistent. This result is opposite to the finding that plants of various species had a greater O<sub>2</sub> sensitivity when grown at elevated CO<sub>2</sub>, suggesting that any potential PO<sub>4</sub><sup>3-</sup> limitation had been removed (Sage, 1989; Spencer and Bowes, 1986). Makino (1994) subsequently questioned whether these observations were due to an increase in absolute capacity to regenerate PO<sub>4</sub><sup>3-</sup>, or a relative decrease in carboxylation and RuBP-regeneration capacities due to increased leaf area, increased dry mass or other factors. On balance, the O<sub>2</sub> sensitivity and A<sub>70</sub>:A<sub>36</sub> results here indicate that while there is partial limitation of photosynthesis at 70 Pa by PO<sub>4</sub><sup>3-</sup> on occasions, usually RuBP-regeneration is limiting and there is no consistent effect of growth CO<sub>2</sub> on this.

### 3.4 Results: Responses of parameters derived from $A/pC_i$ curves and the O<sub>2</sub> sensitivity of photosynthesis to long-term growth at 70 Pa CO<sub>2</sub> as a function of leaf N content

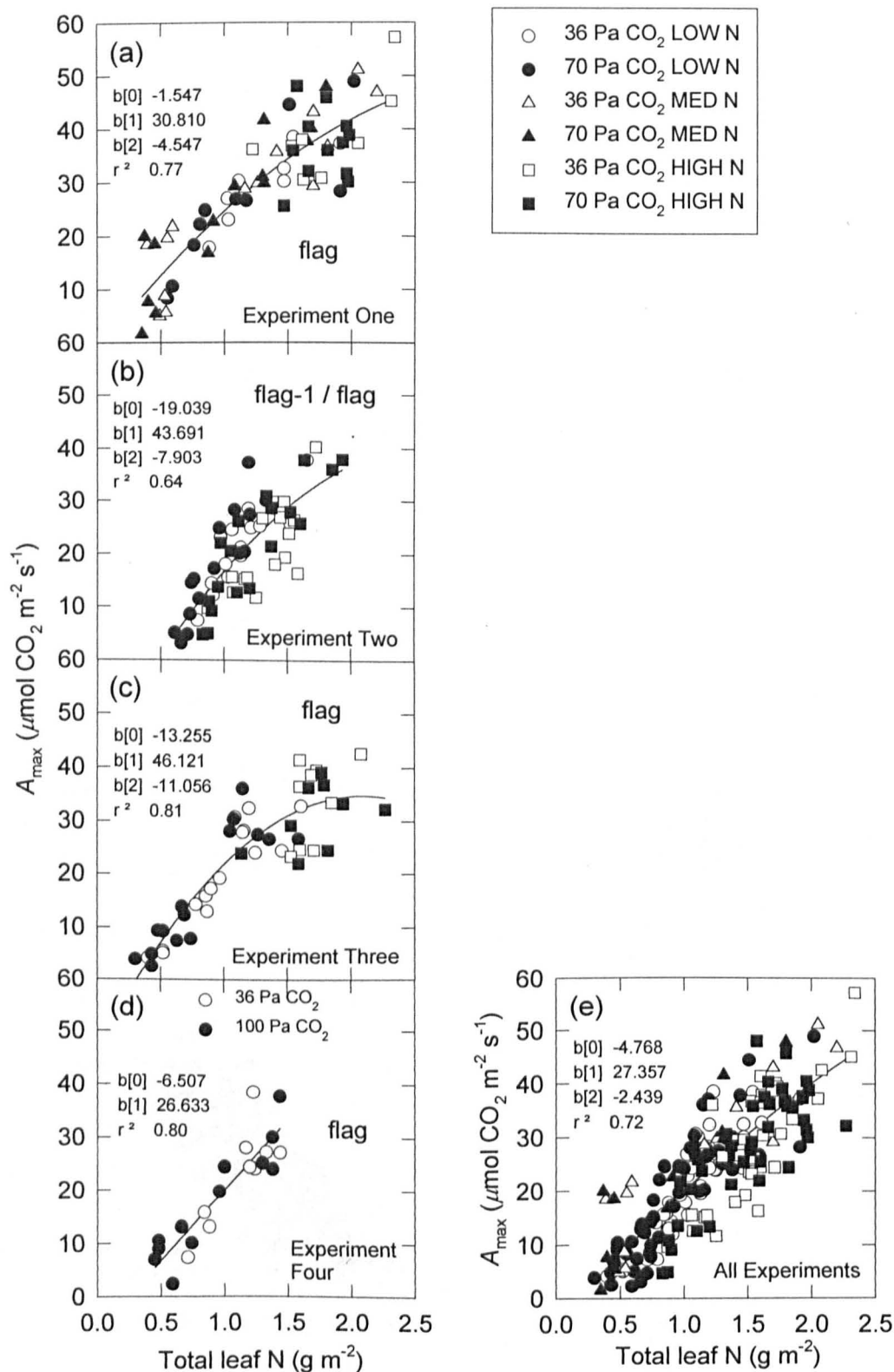
In the previous section it was demonstrated that the general patterns of effects on  $\varepsilon$  (Fig. 3.4) and  $A_{\max}$  (Fig. 3.5) mirrored effects on leaf N content (Fig. 3.3). However, since the points plotted in the above graphs are mean values, it may be that individually plotted values for these parameters as a function of leaf N content measured in the same samples do vary with CO<sub>2</sub> treatment. For example, if as the results of Figure 3.6 suggest, there is a re-balancing between *in vivo* capacities at elevated CO<sub>2</sub>, then on a N content basis per unit area, we might expect to observe changes in these relationships.

For all four experiments the relationship between  $\varepsilon$  and leaf N content was approximately linear, and independent of both N and CO<sub>2</sub> treatments (Fig. 3.8 a-d). In Experiment One the y-intercept of the regression line was not significantly different from zero (0.2  $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1} \text{ Pa}^{-1}$ ) and so has been forced through the origin. For remaining experiments there was a positive intercept on the x-axis which ranged from 0.1 to 0.25 g N m<sup>-2</sup>, suggesting that as total leaf N content increased there was a greater than proportional increase in  $\varepsilon$ . Importantly, this implies that during leaf senescence,  $\varepsilon$  declines faster than leaf N content as a whole.

For Experiments One to Three, the relationship between  $A_{\text{max}}$  and leaf N content was curvilinear (Fig. 3.9 a-c), whilst linear in Experiment Four (Fig. 3.9 d), due to a narrower range of leaf N contents. As for  $\varepsilon$ , in all experiments the relationship between  $A_{\text{max}}$  and leaf N content was independent of N and CO<sub>2</sub>, and for all, there was a positive intercept on the x-axis ranging from 0.07 to 0.5 g N m<sup>-2</sup>. Thus for all experiments, the increase in  $A_{\text{max}}$  was approximately linear and greater than proportional with N up to a leaf N content of about 1.5 g m<sup>-2</sup>.



**Figure 3.8** Carboxylation efficiency versus total leaf N content measured in samples of flag leaves (Experiments One, Three and Four) or a combination of flag and flag-1 (Experiment Two), from full emergence through to senescence. In Experiments One to Three plants were grown at either 36 or 70 Pa  $\text{CO}_2$ , and 36 or 100 Pa  $\text{CO}_2$  in Experiment Four. Different nitrogen applications during growth are indicated in the key. All panels show first order linear regressions and the “All Experiments” panel (e) shows the combined data from all four experiments. For further details see text.



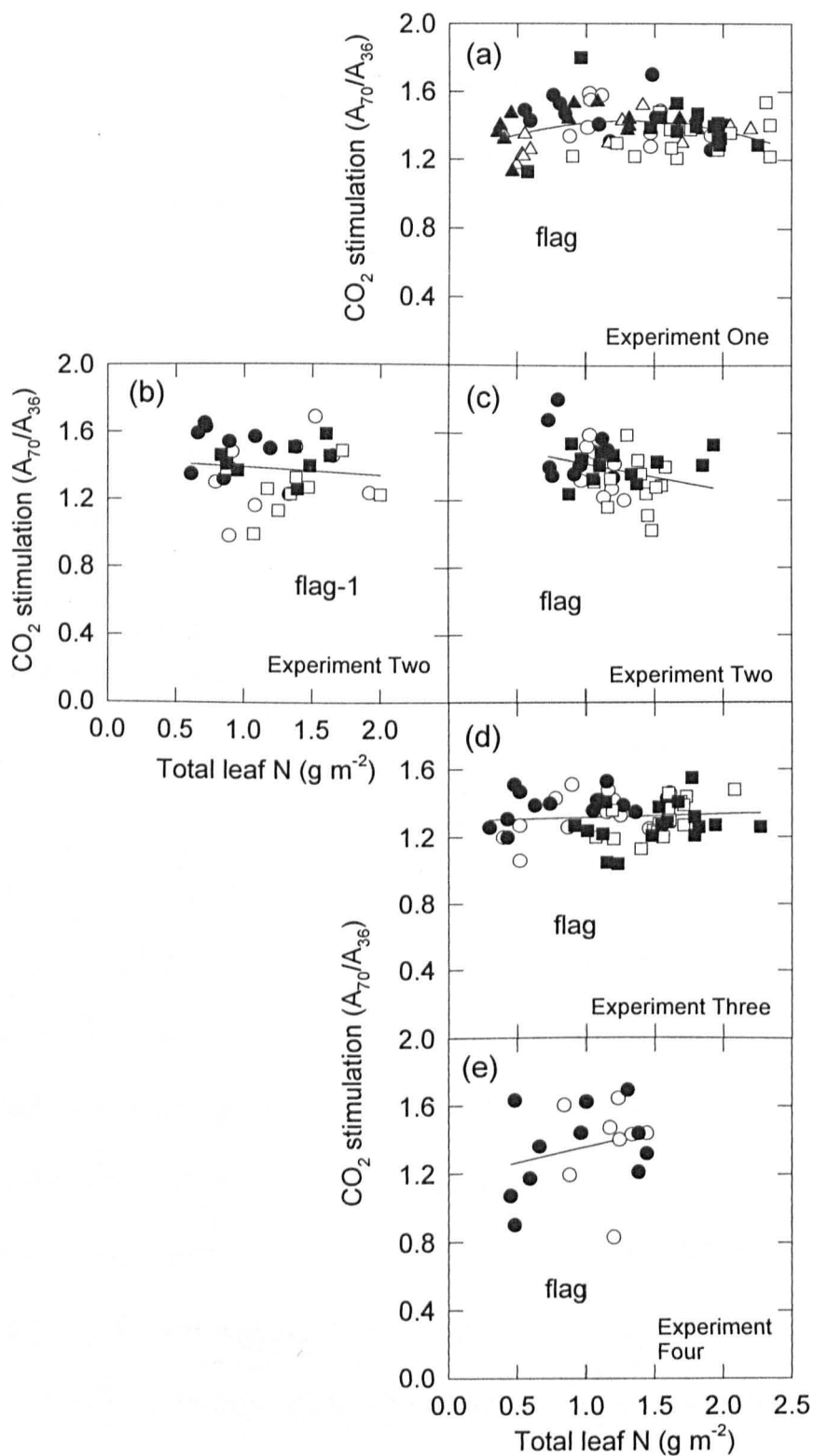
**Figure 3.9**  $A_{\max}$  versus total leaf N content measured in samples of flag leaves (Experiments One, Three and Four) or a combination of flag and flag-1 (Experiment Two), from full emergence through to senescence. In Experiments One to Three plants were grown at either 36 or 70 Pa  $\text{CO}_2$ , and 36 or 100 Pa  $\text{CO}_2$  in Experiment Four. Different nitrogen applications during growth are indicated in the key. All panels show polynomial regressions except for Experiment Four (d), where a linear regression gave the best fit. The “All Experiments” panel (e) shows the combined data from all four experiments. For further details see text.



Thereafter, curvilinearity in the relationship was introduced so that with further increases in leaf N content  $A_{\max}$  was proportionately less stimulated.

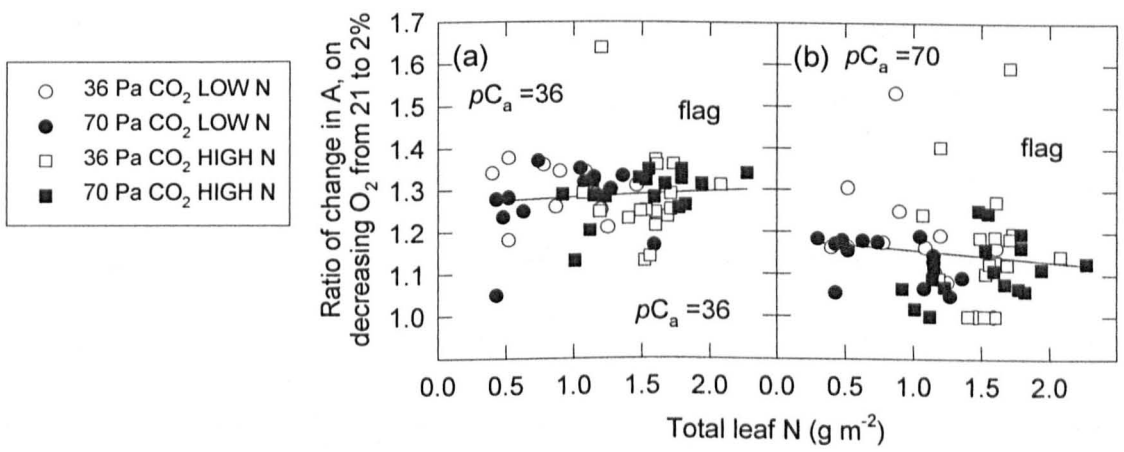
For the relationship between CO<sub>2</sub> stimulation ( $A_{70}:A_{36}$ ) and leaf N content (Fig. 3.10, a-e) responses for all four experiments were independent of the N treatment applied during growth, and, in contrast to the expression of mean values through time (Fig. 3.6), independent of CO<sub>2</sub> as well. However, despite this lack of a significant CO<sub>2</sub> effect, there was a slight trend for higher absolute ratios in elevated CO<sub>2</sub> grown plants as illustrated for a few data points in Experiments One and Two, where ratios increased to between 1.7 and 1.8.

There is evidence that the CO<sub>2</sub> stimulation ratio may actually increase slightly as a function of leaf N content. This is best illustrated in the data for flag leaves of Experiment Two, where with a decrease in leaf N content from 1.9 to 0.7 g N m<sup>-2</sup>, which would occur during senescence, the CO<sub>2</sub> stimulation ratio correspondingly increased from 1.25 to 1.5. A similar response was seen for flag-1 leaves of this same experiment, and to some degree, in the results of Experiment One, although here a polynomial regression was plotted as it gave the best fit, and so subsequently, the CO<sub>2</sub> stimulation ratio only increases with a decline in leaf N content from 2.3 to 1.25 g N m<sup>-2</sup>. In contrast, the ratio was independent of leaf N content in flag leaves of experiment Three, and actually declined with decreasing leaf N content in flag leaves of Experiment Four, although compared to other experiments, far fewer data points were available and the scatter was high.



**Figure 3.10**  $\text{CO}_2$  stimulation ( $A_{70}/A_{36}$ ) versus total leaf N content measured in samples of flag leaves (Experiments One, Three and Four) or a combination of flag and flag-1 (Experiment Two), from full emergence through to senescence. In Experiments One to Three plants were grown at either 36 or 70 Pa  $\text{CO}_2$ , and 36 or 100 Pa  $\text{CO}_2$  in Experiment Four. Different N applications during growth are indicated in the key. For Experiment One (a) and All Experiments (e) polynomial regressions were fitted to the data, and in all other experiments, linear regressions, as these respectively gave the best fits. For further details see text.

Figure 3.11 shows the relationship between the stimulation of photosynthesis on decreasing the  $O_2$  concentration from 21 to 2 kPa against leaf N content for flag leaves of Experiment Three and measured at  $pC_a = 36$  or 70 Pa  $CO_2$ . There was no significant effect of the N treatment applied during growth or  $CO_2$  enrichment on this relationship. Further, when measured at  $pC_a = 36$ , the mean stimulation was approximately 29% and was independent of total leaf N content. At  $pC_a = 70$ , the mean stimulation decreased slightly from 18 to 12%, with increasing leaf N content from 0.3 to 2.3  $g\ m^{-2}$ . This could be interpreted as evidence to suggest that as total leaf N content increased, the increase in the *in vivo* capacity to regenerate  $PO_4^{3-}$  did not increase in proportion with the capacity to regenerate RuBP.



**Figure 3.11** Ratio of the change in photosynthetic rate on decreasing the  $[O_2]$  from 21 to 2kPa versus total leaf N content determined at a  $pC_a$  of 36 (a) or 70 (b) Pa, and measured in samples of flag leaves from Experiment Three, from full emergence through to senescence. Plants were grown at either 36 or 70 Pa  $CO_2$ . Different N applications during growth are indicated in the key. For further details see text.

### Discussion

The results for the relationships of  $\epsilon$ ,  $A_{max}$  and  $CO_2$  stimulation ( $A_{70}:A_{36}$ ) against leaf N content did not depend on growth  $CO_2$  but did depend on leaf N content. As discussed in section 3.3, it seems probable that  $CO_2$  induced increases in

grain number resulted in greater sink strength and a subsequent faster re-mobilisation of N from flag and other remaining leaves. Therefore, the apparent effects of growth at elevated CO<sub>2</sub> on  $\epsilon$ ,  $A_{\max}$  and CO<sub>2</sub> stimulation ( $A_{70}:A_{36}$ ) as a function of time (Figs 3.4, 3.5 and 3.6) can be explained solely by the fact that at a given measurement occasion subsequent to anthesis, 70 Pa CO<sub>2</sub> grown leaves compared to 36 Pa CO<sub>2</sub> grown, contained lesser amounts of N, and therefore lower values for  $\epsilon$  and  $A_{\max}$  or slightly increased CO<sub>2</sub> stimulation ratios ( $A_{70}:A_{36}$ ).

This same conclusion was reached by Nakano et al., (1997) who grew rice at 36 and 100 Pa CO<sub>2</sub> and three N supplies, and similarly found no effect of CO<sub>2</sub> enrichment or the amount of N applied during growth, on the relationships between parameters derived from the  $A/pC_i$  response and leaf N content. One specific parameter these authors measured was the ratio of  $A$  measured at a  $pC_i$  of >60 versus  $A$  measured at a  $pC_i$  of 20, which is similar to, but not exactly the same as the CO<sub>2</sub> stimulation ratio of  $A_{70}:A_{36}$  used in this study, but nevertheless is likewise a good indicator of the shape of the  $A/pC_i$  response, and for which these authors found CO<sub>2</sub> exerted no effect. However, as observed in Experiment Two and to some extent in Experiment One, Nakano et al., (1997) also found that this ratio increased with decreasing leaf N content. Similarly, Makino et al., (1994) observed the same response in an experiment investigating the effect of varying leaf N content on the relative balance between component capacities of photosynthesis in rice.

In considering further that leaf N content can account for the apparent effects of CO<sub>2</sub> enrichment on CO<sub>2</sub> stimulation as a function of time, it is interesting to note that the highly significant ( $P<0.001$ ) effects seen in Experiments One and Two (Fig. 3.6 a-c), and less significant effect in Experiment Three and totally absent in

Experiment Four, correlates with the change in CO<sub>2</sub> stimulation ratio with leaf N content seen in the former but not the latter experiments (Fig. 3.10 a-e). This also explains why significant effects of N treatments applied during growth on CO<sub>2</sub> stimulation were observed for Experiments One and Two, but not Three and Four, as discussed in the results of section 3.3.

The suggestion that the *in vivo* capacity to regenerate PO<sub>4</sub><sup>3-</sup> did not increase in proportion with the capacity to regenerate RuBP (Fig 3.10 b), is supported by Makino et al (1994), who measured activities of cytosolic FBPase and SPS in rice, and concluded that the capacity to synthesise sucrose increased to a lesser extent than that of Rubisco and cytochrome *f* at both *in vivo* and *in vitro* levels, with increasing leaf N content.

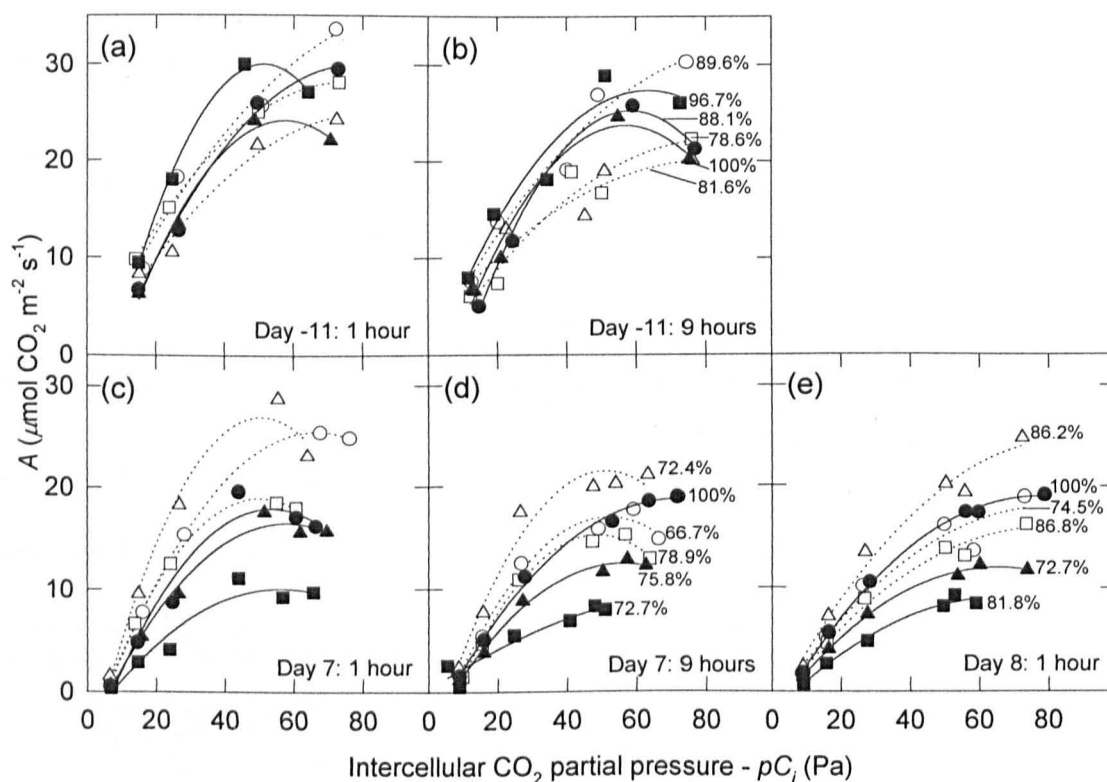
### 3.5 The effect of CO<sub>2</sub> enrichment on the depression of photosynthesis through the photoperiod

#### *Introduction*

In section 3.2.3 it was clearly demonstrated for a selection of 36 Pa CO<sub>2</sub> grown plants, that photosynthesis at high  $pC_i$  was progressively depressed through the course of the photoperiod, but fully recovered by the start of the next. This effect has previously been correlated with an increased accumulation of carbohydrates within leaves, resulting in a decreased capacity to regenerate PO<sub>4</sub><sup>3-</sup> (Azcón-Bieto, 1983), followed by mobilisation during the dark period to result in a full recovery by the start of the next photoperiod. Since CO<sub>2</sub> enrichment stimulates photosynthesis, at least always in the short-term, photosynthesis in leaves grown at 100 compared to 36 Pa CO<sub>2</sub> might be depressed to a greater degree towards the end of the photoperiod,

correlating with a proportionately greater accumulation of carbohydrates. To test this hypothesis,  $A/pC_i$  responses of flag leaves from Experiment Four grown at either 36 or 100 Pa  $\text{CO}_2$  were measured at one and nine hours into the photoperiod on two separate occasions, 11 days prior and 7 days after anthesis. The method and materials used were the same as those described in section 3.2.3.

## Results



**Figure 3.12** The effect of different measurement times in the photoperiod on the  $A/pC_i$  response in flag leaves of wheat from Experiment Four, grown at a low N supply and 36 or 100 Pa  $\text{CO}_2$ , and measured on two occasions at -11 and 7 days after anthesis. The  $A/pC_i$  response was determined in the same leaves at 1 hour into the photoperiod (a & c), 10 hours into the photoperiod (b & d), and 1 hour into the photoperiod the following day after a 10 hour dark period (e). Values shown are the percentage  $A_{\text{max}}$  of the  $A_{\text{max}}$  obtained at 1 hour on day one, for each replicate leaf, where  $n = 3$ . For further details see text.

For leaves measured on the first occasion at 11 days prior to anthesis (Fig.

3.12, a & b), photosynthesis at high  $pC_i$  and saturating light was depressed on average, to 83.3 % ( $\pm 3.3$ ;  $P < 0.01$ ) and 94.9 % ( $\pm 3.5$ ; NS) of rates at the start of the photoperiod, for 36 and 100 Pa CO<sub>2</sub> grown plants respectively. However, at 9 hours there was no significant difference between the magnitude of depression in  $A_{\max}$  between leaves of plants grown at 36 or 100 Pa CO<sub>2</sub>.

When measured 7 days after anthesis (Fig. 3.12, c-e), for 36 Pa CO<sub>2</sub> grown leaves after 9 hours of illumination,  $A_{\max}$  was significantly ( $P < 0.01$ ) lowered to 72.6% ( $\pm 3.5$ ) of that at the start of the day, and this recovered slightly but non-significantly to 82.5% ( $\pm 4.0$ ) following 10 hours in darkness. Similarly, for 100 Pa CO<sub>2</sub> grown leaves,  $A_{\max}$  was lowered, but surprisingly to a lesser degree and with no significance, to 82.8% ( $\pm 8.6$ ), and this depression was only very slightly, but non-significantly, alleviated to 84.8% ( $\pm 8.0$ ) following 10 hours in darkness. At nine hours and one hour on day two, there was again, no significant difference between the magnitude of depression in  $A_{\max}$  between leaves of plants grown at 36 or 100 Pa CO<sub>2</sub>.

### *Discussion*

For the results presented above, there was clearly no difference in the response of the depression of  $A_{\max}$  through the photoperiod, in flag leaves of wheat grown at either 36 or 100 Pa CO<sub>2</sub>. If, as the results of Azcón-Bieto (1983) suggest, the degree of depression correlates with the degree of carbohydrate accumulation, then this implies, since the rate of CO<sub>2</sub> fixation and therefore rate of carbohydrate production was greater at 100 Pa CO<sub>2</sub>, that the capacity to transport assimilate or store carbohydrate as starch in chloroplasts or soluble sugars within vacuoles, was

increased in plants grown at 100 Pa CO<sub>2</sub>. Given that biomass and final grain yield was stimulated at elevated CO<sub>2</sub> (section 2.10), it is not unreasonable to assume the former, and that carbohydrate was readily transported due to increased sink demand. Alternatively, one could suggest that the additional carbohydrate at 100 Pa CO<sub>2</sub> was transported and stored in the stems, although from the results for carbohydrate content of these organs measured at anthesis in two experiments and presented in Figure 2.6, no evidence for increased accumulation at elevated CO<sub>2</sub> was found. Also, for these same data, no differences were found for the concentration of carbohydrate within leaves of 36 and 70 Pa CO<sub>2</sub> grown plants at anthesis, which supports the assumption here, that the concentrations of carbohydrate in leaves were not significantly different between CO<sub>2</sub> treatments at the end of the photoperiod on the days measured.

### **3.6 The effect of CO<sub>2</sub> enrichment on the accumulation of soluble carbohydrate in flag leaves of spring wheat**

#### *Introduction*

As outlined in Chapter One, growth of plants for prolonged periods at elevated  $pC_a$  may result in a decrease in their capacity for photosynthesis leading to photosynthetic acclimation (Peet et al., 1986; Sage et al., 1989; Yelle et al., 1989), and this has frequently been associated with increased starch and soluble carbohydrate concentrations in source leaves (Delucia et al., 1985; Stitt, 1991; Long and Drake, 1992). Several lines of evidence have suggested that the regulation of the expression of photosynthetic genes, via increased soluble carbohydrate concentration, may underlie suppression of photosynthesis at elevated CO<sub>2</sub> (Sheen, 1989; Stitt, 1991;



Krapp et al., 1993; Webber et al., 1994). Consequently, to determine whether any CO<sub>2</sub> effects on photosynthesis and loss of components from leaves which might be observed would parallel changes in carbohydrate concentration, this parameter was measured on plants grown at either 36 or 100 Pa CO<sub>2</sub> in Experiment Four. The pattern of leaf carbohydrate concentration in samples taken from plants under growth conditions was followed on five occasions through ontogeny for flag leaves, and through the course of the photoperiod on one occasion at nine days after anthesis during the period of rapid grain filling. Specifically it was hypothesised that if a long-term accumulation of carbohydrates does lead to photosynthetic suppression, then the best time in the diurnal cycle to look for an increase in the residual carbohydrate concentration will be at the start of the photoperiod. This is because any large increases in concentration above a basal level to supply respiration, would represent that carbohydrate which the plant was unable to re-mobilise during the dark period, and this would combine with carbohydrate produced in the subsequent photoperiod and hence, since plants were growing under constant and controlled conditions, lead to a net accumulation with time.

### *Materials and Methods*

Flag leaves were harvested under growth conditions during the first hour of the photoperiod on days -7, 0, 3, 7, 9 and 16 relative to anthesis, and then 40 mm above the ligule and 40 mm below tips were removed, and leaves immersed and stored in liquid N<sub>2</sub>. In the diurnal experiment, samples were taken at 1, 3, 5.5, 9 and 13.5 hours into the 14 hour photoperiod, and sampled in the same way.

Leaf samples of measured area were extracted in a pestle and mortar on ice

containing 2 mL of 100 mM Hepes buffer (pH 7.4), 20 mM MgCl<sub>2</sub> and 1 mM EDTA. Then 400  $\mu$ L of extract was immediately boiled in 600  $\mu$ L of ethanol for 20 minutes to denature carbohydrate degrading enzymes, and then subsequent to cooling, contents were clarified at 12 000 xg for 3 min and the supernatant stored at -20°C.

Glucose, fructose and sucrose were measured via the reduction of NADP by glucose-6-phosphate dehydrogenase after the sequential addition of hexokinase, phosphoglucose isomerase and invertase (Jones et al., 1977). The method was adapted for use on an ELISA plate, using reduced volumes and an ELISA plate reader to measure absorbance changes (Viola and Davies, 1992; Paul and Driscoll, 1997).

### *Results*

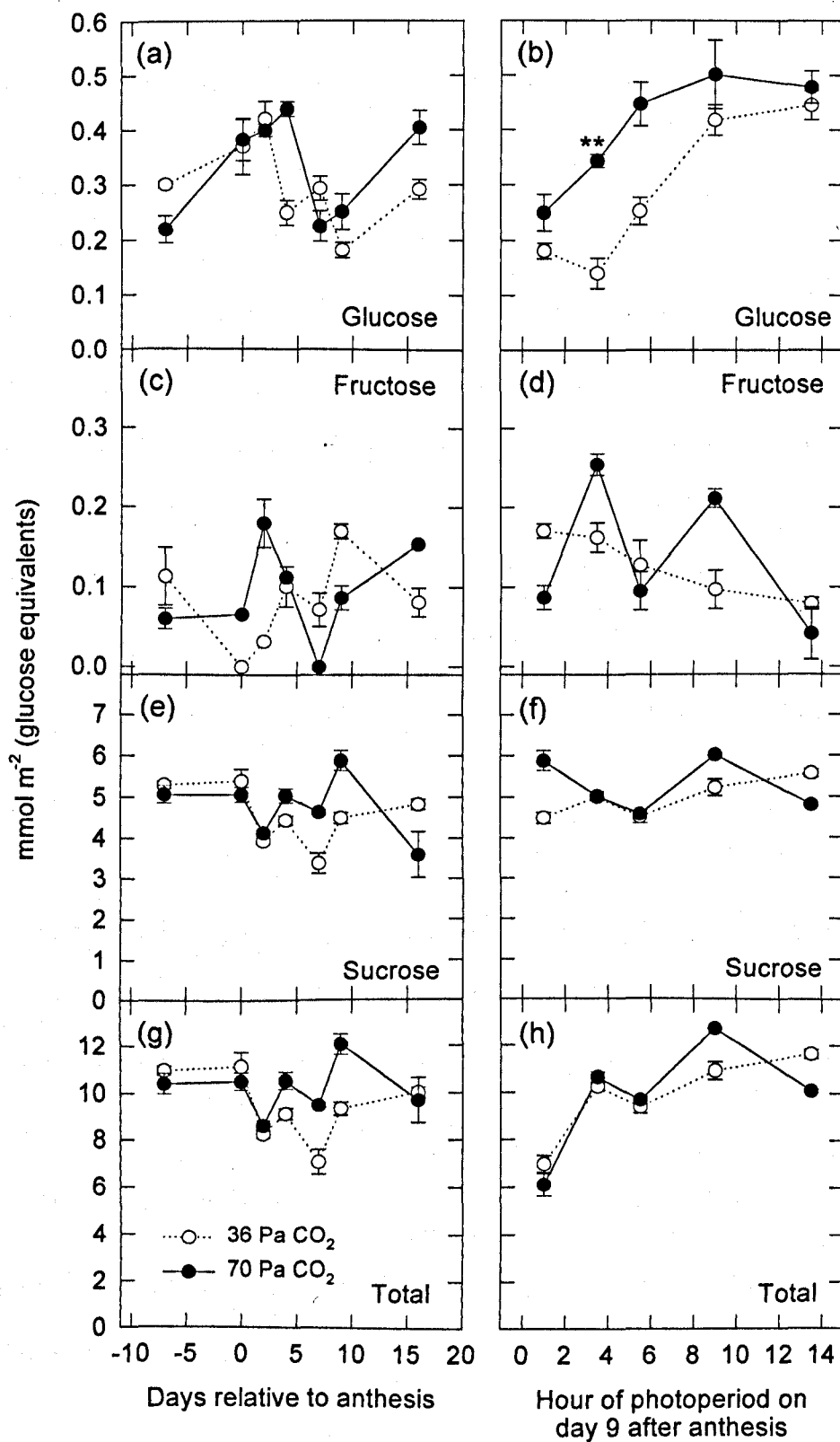
Absolute values for leaf carbohydrate concentration were similar in range to those reported elsewhere for spring wheat, as were the relative ratios of glucose:fructose:sucrose obtained (Morcuende et al., 1996). Figure 3.13 (a, c, & e) below shows that there was no significant effect of growth at 100 Pa CO<sub>2</sub> on the concentration of glucose, fructose or sucrose in flag leaves measured at the beginning of the photoperiod and on occasions from full emergence through to senescence. Similarly, there was no significant CO<sub>2</sub> effect on the rate of accumulation of fructose and sucrose in flag leaves measured through the course of the photoperiod on one occasion at 9 days after anthesis (Fig. 3.13, b, d & f). However, glucose accumulation was more rapid at 100 Pa CO<sub>2</sub> which resulted in a significant ( $P<0.01$ ) increase in glucose concentration at 3.5 hours into the

photoperiod, but this effect was lost towards the end of the day, when glucose concentrations were not significantly different from 36 Pa CO<sub>2</sub> grown controls.

### *Discussion*

That the concentration of glucose, fructose and sucrose in 36 and 100 Pa CO<sub>2</sub>, low N grown flag leaves were never significantly different when measured at the start of the photoperiod on occasions through development, suggests that sinks in 100 Pa CO<sub>2</sub> grown plants, despite the low N status and increased photosynthesis, had developed additional and sufficient sink capacity to remove any accumulation of carbohydrates overnight. The same observation and conclusion was made for wheat grown at 55 Pa CO<sub>2</sub> under FACE, where additionally starch and fructans were measured (Nie et al., 1995b).

Further, it is apparent from the diurnal analysis at 9 days after anthesis, that at the end of the photoperiod there were no significant differences in the concentrations of glucose, fructose or sucrose between 36 and 100 Pa CO<sub>2</sub> grown flag leaves. Since the concentration of glucose had increased more rapidly in 100 Pa CO<sub>2</sub> grown flag leaves at the start of the photoperiod, these results in combination, tentatively suggest that the rate of export of these particular carbohydrates, or their conversion and storage as fructans in vacuoles, had increased at 100 Pa CO<sub>2</sub>. This suggestion is supported by the data presented in the previous section, where it was demonstrated that for flag leaves of the same experiment, the magnitude of depression of photosynthesis at high  $pC_i$  at the end of the photoperiod was not significantly different between 36 and 100 Pa CO<sub>2</sub> grown leaves



**Figure 3.13** The response of various carbohydrate concentrations through time (a, c, e & g) or during the photoperiod (b, d, f & h), to long-term growth at either 36 or 100 Pa  $\text{CO}_2$  in low N grown flag leaves of wheat. For the response through time, samples were always taken within an hour following the end of the dark period. Bars = 1x Std Err, and  $n = 6$ . Asterisks denote significant effects of  $\text{CO}_2$  for the occasion indicated: \*\* = ( $P < 0.01$ ). For further details see text.

If 100 Pa CO<sub>2</sub> grown leaves had accumulated more carbohydrate during the photoperiod, then one might expect the depression in photosynthesis to be greater, but this was not the case.

This lack of evidence for a long-term accumulation of carbohydrate in leaves grown at 100 Pa CO<sub>2</sub>, suggests that these plants were able to utilise all the additional assimilate. This implies that there was no source-sink imbalance at 100 Pa CO<sub>2</sub>, which would explain why no evidence was found in section 2.10 for a change in the allocation patterns of carbon and N at the whole plant level, for Experiments One and Three.

### 3.7 Conclusions

For results expressed on a leaf area basis, long-term growth of spring wheat at elevated concentrations of CO<sub>2</sub> had no significant effect on photosynthetic rate or any other parameter derived from  $A/pC_i$  responses at full emergence, in any leaf of any experiment, and on no subsequent occasion for flag-2 leaves. Significant effects were however seen on occasions at (flag-1 leaves), or subsequent (flag leaves) to anthesis, in which  $\varepsilon$  and  $A_{\max}$  declined faster at elevated CO<sub>2</sub> and especially at low N, and this response tended to persist for the remainder of the grain filling period. Further, an increase in the CO<sub>2</sub> stimulation ratio ( $A_{70}:A_{36}$ ) on some occasions late in ontogeny and especially in low N grown plants, indicated that some partial rebalancing between carboxylation and RuBP-regeneration capacities may have occurred. Additionally, PO<sub>4</sub><sup>3-</sup> regeneration capacity was never completely limiting at growth  $pC_a$ , indicating that there was no decrease in RuBP-regeneration capacity

relative to  $\text{PO}_4^{3-}$  regeneration capacity.

However, when these parameters were expressed as a function of leaf N content, no significant effects of  $\text{CO}_2$  enrichment on any of these relationships was found. Thus in conclusion and contrary to the hypothesis being tested, from 675  $A/pC_i$  responses of four separate experiments, there was no evidence for a change in the *in vivo* balance between capacities determining photosynthesis in leaves of plants grown long-term at elevated  $\text{CO}_2$ , compared to those currently grown at 36 Pa  $\text{CO}_2$ . This indicates that the full potential increase in  $\text{CO}_2$  fixation to be gained at elevated  $\text{CO}_2$  was not realised. Nevertheless, there was an indirect  $\text{CO}_2$  effect, which due to a stimulation of ear size and grain number (Table 2.9) which resulted in increased sink-strength, led to a faster loss of N and therefore photosynthetic capacity from flag-1 and flag leaves of plants grown at elevated  $\text{CO}_2$  and particularly at low N. This accounts for the effects seen when results were related to treatments rather than leaf N content.

Thus, in terms of the main hypothesis of biochemical optimisation being tested in this study, there is at this stage, little evidence to support its occurrence. However, as outlined in the introduction to this chapter, gas exchange analysis is merely a tool to assess *in vivo* capacities, and is based upon a number of assumptions outlined in the von Caemmerer and Farquhar (1981) model of  $\text{C}_3$  photosynthesis. Therefore, because changes in the amounts of biochemical components which determine capacities could still be occurring, but being masked by changes in activation state, it is necessary to measure the activity and amount of components directly, and this task forms the topic of discussion in the next two chapters. Further, such analysis provides the opportunity to test and validate some of the

assumptions of the  $C_3$  model.





## CHAPTER 4

### The long-term effect of elevated partial pressures of CO<sub>2</sub> on the amount and activation of Rubisco in Spring Wheat

#### 4.1 Introduction

The model of biochemical acclimation to elevated CO<sub>2</sub> (Makino, 1994; Webber, 1994; Medlyn, 1996) discussed in Chapter One, suggests that with long-term exposure to double the existing atmospheric partial pressure of CO<sub>2</sub> (36 Pa), the amount of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) could be decreased within leaves by as much as 30% for a given N content, with no deleterious effect on photosynthetic rate. If this biochemical acclimation were to operate, this would benefit N-use efficiency, resulting in increased N availability for use elsewhere within the plant. Indeed, when results are expressed on a leaf area basis, there is considerable evidence that for plants grown under such conditions, the amount of Rubisco per unit leaf area does decrease (Peet et al., 1986; Sage et al., 1989; Rowland-Bamford et al., 1991; Jacob et al., 1995). However, it has also been demonstrated that growth at elevated  $pC_a$  may also decrease total leaf N content per unit leaf area (Rowland-Bamford et al., 1991; Conroy and Hocking, 1993; Tissue et al., 1993; Delgado et al., 1994; Rogers et al., 1996), so that decreases in Rubisco must be measured in relation to total leaf N content if meaningful conclusions are to be drawn. To date this has only been done in a limited number of studies on pea and wheat (Makino, 1994) and rice (Nakano, 1997). In these examples, plants were grown hydroponically under controlled conditions at 35 or 70 Pa CO<sub>2</sub> and analysed on only one occasion at full leaf expansion. No differences between the two CO<sub>2</sub> treatments were found for any of the relationships between amounts of Rubisco (or

other components of the photosynthetic machinery) and leaf N content, suggesting that these species do not have an ideal acclimation to elevated CO<sub>2</sub> at the biochemical level (Makino, 1994). However, these results do not rule out the possibility that a response might develop later in leaf ontogeny. In flag leaves of wheat, while no differences between CO<sub>2</sub> treatments were observed at full emergence, there was an apparent more rapid decline in Rubisco content at elevated  $pC_a$  (Nie et al., 1995a).

Further, evidence from Sage et al., (1989) has demonstrated that whilst Rubisco activation in bean plants declines immediately after transfer to elevated CO<sub>2</sub> as one might expect, the level of activation continues to remain low, even throughout a subsequent four week exposure period. It is argued that if plants do have an ideal biochemical acclimation to elevated CO<sub>2</sub>, then in the long-term, Rubisco activation should recover as the excess of this protein is degraded (Makino, 1994). The evidence for beans however, suggests that biochemical acclimation to elevated CO<sub>2</sub> may be limited.

Thus, the major aim of the work described in this chapter, was to determine whether or not an ideal biochemical acclimation to elevated CO<sub>2</sub> occurs in leaves of spring wheat, either at full leaf expansion or any subsequent stage of ontogeny, with the main focus of attention being on the response of the amount and activation of Rubisco. To this end, the following pages describe the development and testing of assays for measuring these parameters. Data from four separate experiments relating to the long-term effects of elevated CO<sub>2</sub> on the amounts of Chl, soluble protein and the amount and activity of Rubisco are then presented, first on a leaf area basis as a function of leaf age, and then on a leaf area basis as a function of total leaf N content. Finally, conclusions are drawn as to whether or not wheat has an ideal

biochemical acclimation to elevated CO<sub>2</sub>, based on the response of Rubisco to this treatment.

## 4.2 Development of an Extraction Protocol for Rubisco

In this section, single, brief experiments are described for the development and optimisation of a method for extracting Rubisco from leaf material of varying age, upon which assessment of the amount and activity of this protein were to be determined.

### 4.2.1 *The Effect of centrifugation time on Rubisco recovery*

#### *Introduction*

Plant tissues contain a wide range of proteins which vary greatly in their properties, and require specific conditions for their extraction and purification (Shewry and Fido, 1996). One aspect of particular importance to this study is the length of time given to the recovery of protein during centrifugation, as insufficient centrifugation may not fully remove cellular debris and other contaminants which may cause interference at later stages of assay. Further, in the literature, recommended centrifugation times for the recovery of Rubisco from wheat leaves using similar extraction procedures to those employed in this study, range from three (Delgado et al., 1994) to five minutes (Evans, 1983; Makino et al., 1992; McKee and Woodward, 1994), so that some investigation was considered necessary to determine optimal centrifugation parameters. Further, there have been reports in the literature describing the association of Rubisco with thylakoid membranes (Akazawa, 1978;

McNeil and Walker, 1981; Mori et al., 1984), so that an additional consideration was that some Rubisco might be associated with fragments of thylakoid membrane concentrated in the pellet, although this potential problem is considered further in section 4.2.3. Thus the aim of this brief and preliminary study was to determine how the length of centrifugation within a time span of 0.5 to 5 min would affect the recovery of Rubisco.

### *Materials and Method*

Freshly harvested flag-1 (leaf preceding flag) leaves of wheat (413 mm<sup>2</sup> in total) were combined, finely chopped and ground in a pestle and mortar on ice, in 10 mL of a 50 mM Bicine buffer (pH 8.0), containing 20 mM MgCl<sub>2</sub>, 1 mM EDTA and 50 mM  $\beta$ -Mercaptoethanol. The homogenate was divided equally between five microcentrifuge tubes and centrifuged at 10000 xg (4°C), with tubes being sequentially removed after a half, one, two, three and five minutes. Supernatants were transferred to clean microcentrifuge tubes, and aliquots removed for the determination of Chl and soluble protein content (see sections 2.4 and 2.5 respectively). From the remaining supernatant, 500  $\mu$ g of soluble protein was removed and added to 20  $\mu$ L of SDS solubilisation buffer (250 mM Tris buffer (pH 7.6) containing 250 mM Dithiothreitol, 10% (w/v) SDS, 0.2% (w/v) bromophenol blue and 10% (w/v) glycerol), 150  $\mu$ L of 20% (v/v) glycerol and the total volume made up to 520  $\mu$ L with distilled H<sub>2</sub>O. Samples were denatured at 90°C for five minutes. Rubisco LSU was quantified by PAGE (see section 2.6 for details) against wheat Rubisco standard which was a gift from Dr M.A.J Parry (purification described in Gutteridge et al., (1982)), and resultant bands scanned using a

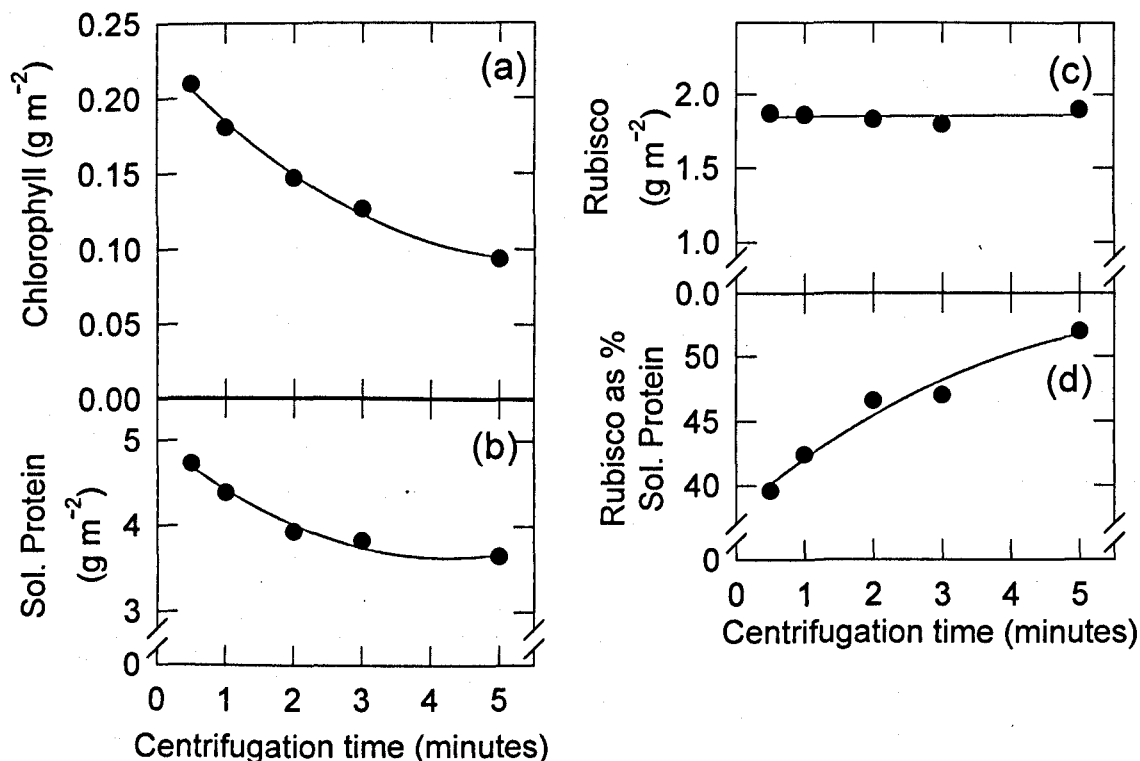
densitometry software package (SigmaGel, Jandel Scientific, Sausalito, CA), from which total Rubisco was estimated assuming eight SSUs represent 24% of the intact holoenzyme (L<sub>8</sub>S<sub>8</sub>) by mass.

### *Results*

Figure 4.1 (a-d) below, shows the relationships between the amounts of Chl, soluble protein and Rubisco in sub-samples of the same homogenate centrifuged for different lengths of time. With increased centrifugation from a half to five minutes, the amount of Chl (Fig. 4.1 a) recovered was decreased by 55% and soluble protein (Fig. 4.1 b) by 24%, although the rate of loss in the latter rapidly tailed off between two and five minutes. In contrast, the amount of Rubisco recovered (which was estimated taking into account the volume of supernatant removed containing 500  $\mu$ g soluble protein and which would vary between sub-samples) remained largely unaffected by increasing centrifugation times.

### *Discussion*

The substantial decline in the amount of Chl recovered with increased centrifugation time most likely reflects increased sedimentation of fragmented thylakoids within which Chl is bound to intrinsic proteins. However, since remaining estimations of Chl reported in other sections of this work were determined at the crude homogenate stage prior to centrifugation, the findings here for Chl are of interest, but of no consequence to this study.



**Figure 4.1** The effect of increased centrifugation time (at  $10000 \times g/4^{\circ}\text{C}$ ) on the recovery of Chl, soluble protein and Rubisco extracted from leaf material of *T.aestivum* L.cv Minaret. For further details see text.

In contrast, the 20% decline in soluble protein content with centrifugation times from a half to two minutes, during which the amount of measured Rubisco remains largely unaffected, has implications for the reliability of expression of the latter as a fraction of the former, as used in some studies (Wittenbach, 1979; Evans and Austin, 1986), since the relationship clearly varies with centrifugation time (Fig. 4.1 d). Given that the recovery of Rubisco is unaffected, a likely explanation is that extrinsic proteins attached to membrane fragments which become sedimented with increased centrifugation (as for Chl), amplify estimations of soluble protein, until a sufficient length of centrifugation has taken place to remove them. Over-estimation can occur, because the binding of Coomassie brilliant blue G-250 used in the soluble protein assay (see 2.5 for details) does not discriminate between true soluble proteins and those attached to, or embedded in membranes (Bradford, 1976). Thus, in

subsequent assays where estimations of 'true' soluble protein content were required, centrifugation times of at least two minutes (at 10000 xg) were used. In contrast, where accurate measurements of soluble protein content were not necessary and there was an increased emphasis for rapid extraction as for activity assays (see section 4.5), shorter centrifugation times were used.

#### 4.2.2 *The effect of protease inhibitors, PVP, length of standing on ice and sample re-freezing on the recovery of Rubisco from samples*

##### *Introduction*

The initial homogenisation of leaf material results in an immediate breakdown of cellular compartmentation, which can make proteins of interest susceptible to proteolytic degradation. The degree of degradation depends upon leaf age, species, the intrinsic susceptibility of the protein of interest, and the degree to which the degradative machinery is disassembled and diluted in extraction (Beynon, 1989). To overcome these problems, a host of chemicals which inhibit proteases have become available and are routinely used in plant extraction protocols, common examples being  $\alpha$ -aminocaproic acid, phenylmethanesulphonyl fluoride (PMSF; an inhibitor of serine proteases) and  $\beta$ -Mercaptoethanol (an inhibitor of metaloproteinases (Beynon and Oliver, 1996) in addition to its presence in buffers as a reducing agent). An additional problem with leaves in many species as they age, is the increased accumulation of phenols and tannins which interfere with purification (Pierpoint, 1996), but can be removed by the addition of natural and synthetic polymers such as insoluble polyvinylpyrrolidone (PVPP) onto which contaminants are adsorbed.

The aim therefore, of this short study, given that the effects of elevated CO<sub>2</sub> on progressively older leaf material was to be investigated, was to analyse how the presence or absence of these chemicals affected the recovery of Rubisco. An additional aim was to determine the length of time for which samples could be left on ice following extraction and clarification without causing the degradative loss of protein, and of the effect of re-freezing and thawing these same samples following extraction, but prior to analysis. This was important from a practical consideration, given the bulk of samples to be analysed and a desire to know working constraints. Mature to senescent material was chosen for analysis in this experiment since this might be expected to contain reasonable concentrations of tannins, phenols and proteases if they were going to be present.

#### *Materials and Method*

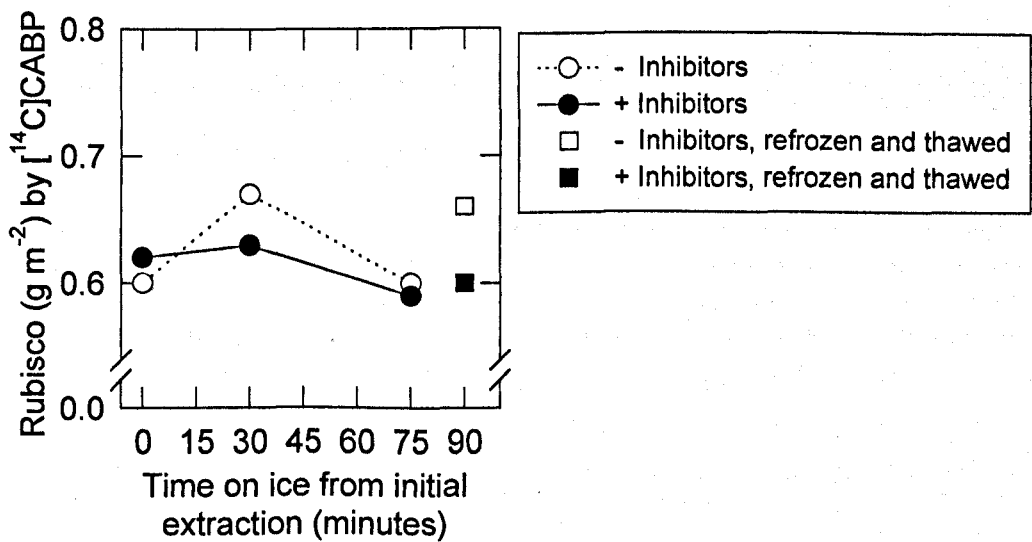
Initially in a cold room, 120 mm<sup>2</sup> of freshly harvested senescent flag leaf material (approximately 20 d post-anthesis) was quickly chopped into 1 mm wide strips, thoroughly mixed and split on a mass basis into two equal sub-samples. Both were placed in individual foil bags and frozen in liquid N<sub>2</sub>. The first sub-sample was ground in a pestle and mortar on ice, in 2 mL of a 50 mM Bicine buffer (pH 8.0), containing 20 mM MgCl<sub>2</sub>, 1 mM EDTA and 50 mM  $\beta$ -Mercaptoethanol, and hereafter referred to as buffer 'A'. Prior to centrifugation at 10000 xg for three minutes at 4°C, two aliquots of the crude homogenate were removed for the determination of Chl. The resulting supernatant was divided into four aliquots of 200  $\mu$ L each, one being immediately re-frozen in liquid N<sub>2</sub>, two being left to stand on ice for 30 and 75 minutes respectively, and the fourth analysed immediately (zero minutes) for Rubisco content using the [<sup>14</sup>C]CABP binding method described below



(see section 4.4). After thawing, or the allotted time on ice, the remaining aliquots were analysed in the same way. The second set of leaf material was treated as above, but for extraction in buffer 'B', which additionally contained protease inhibitors as 10 mM  $\alpha$ -aminocaproic acid, 0.2 mM PMSF and approximately 1% (w/v) of insoluble PVPP to remove phenols and tannins.

Results

Despite a slight decline, standing samples on ice for up to 75 minutes had no significant effect on the amount of Rubisco recovered (Fig. 4.2), for which the same non-significant effects of the presence of protease inhibitors and PVPP were also found. Likewise, re-freezing and thawing samples had no significant effect on the recovery of Rubisco.



**Figure 4.2** The effect of standing time on ice, with or without (closed and open symbols respectively) inhibitors of proteases and PVPP present in the extraction buffer, on the recovery and amount of Rubisco measured by the binding of [<sup>14</sup>C]CABP from senescent flag leaf material of wheat. Additionally shown (squares) is the effect of re-freezing and thawing on the same samples, following extraction but prior to assay. For further details see text.

## *Discussion*

These results suggest that proteases, phenols and tannins do not present a significant problem to the recovery of Rubisco in ageing leaves of wheat, although an effect of other classes of proteases or phenols/tannins not inhibited by  $\alpha$ -aminocaproic acid, PMSF,  $\beta$ -Mercaptoethanol or adsorbed by PVPP, can not be completely ruled out. However, in practical terms it is more likely that the increasingly fibrous nature of wheat leaves with maturation, requiring harder and perhaps longer homogenisation, presents more of a potential problem to enzyme recovery, even if proteases not inhibited by the chemicals tested here are present.

Whilst batches of clarified samples were never left on ice beyond 30 minutes or re-frozen and defrosted prior to analysis, it was useful and interesting to know that samples would remain comparatively robust under these conditions. Given these findings, the subsequent inclusion of protease inhibitors (with the exception of  $\beta$ -Mercaptoethanol which was primarily present to prevent oxidation of proteins) and insoluble PVP in the extraction buffer was not considered necessary.

### *4.2.3 The effect of Triton-X-100 on the recovery of Rubisco*

## *Introduction*

Depending on species, it has been reported (Akazawa, 1978) that as much as 27% of total Rubisco may be bound to thylakoid membranes and that this must be solubilised for full extraction and accurate quantification. Triton-X-100 is a non-ionic surfactant which has been shown to be the best of several, in releasing Rubisco from membranes whilst not affecting activity (Makino and Osmond, 1991). In their study, it was observed that the maximum binding of Rubisco to thylakoid

membranes of pea occurred at pH 8.0, but could be fully removed by the addition of Triton-X-100, which was most effective in the range 0.04 to 0.2% by volume. Makino and Osmond (1991) subsequently concluded that accurate quantification of Rubisco in pea requires dilute Triton-X-100 in the homogenate, and that previous estimates of Rubisco as a fraction of leaf protein or N (Evans and Seeman, 1989), may need to be corrected. Given these observations and the nature of this study, the effect of Triton-X-100 on the recovery of wheat Rubisco was investigated.

### *Materials and Method*

Five wheat leaves (each approximately 1200 mm<sup>2</sup>) at full emergence were randomly selected and individually cut into 1 mm wide strips, mixed, split into two sub-samples and frozen in liquid N<sub>2</sub>. One sub-sample for each leaf was ground in a pestle and mortar on ice, in 2 mL of a 50 mM Bicine buffer (pH 8.0), containing 20 mM MgCl<sub>2</sub>, 1 mM EDTA and 50 mM  $\beta$ -Mercaptoethanol, hereafter called buffer 'A'. Prior to centrifugation at 10000 xg for three minutes at 4°C, two aliquots of the crude homogenate were removed for the determination of Chl. Supernatant was transferred to a clean microcentrifuge tube, and aliquots were removed for the determination of soluble protein and Rubisco content, the latter being assayed by the [<sup>14</sup>C]CABP binding assay described below (see 4.4). Remaining sub-samples were analysed in the same manner, but for the addition of 0.2% (v/v) Triton-X-100 to buffer 'A', hereafter referred to as buffer 'B'.

### *Results*

On average there was a slight and surprising 7% decrease in the amount of Rubisco recovered where Triton-X-100 was included in the extraction buffer,

although this effect was not significant across the range of samples measured (Table 4.1).

Sample	Rubisco g m <sup>-2</sup>		Soluble Protein g m <sup>-2</sup>		Rubisco % Sol. Protein	
	- Triton	+ Triton	- Triton	+Triton	- Triton	+ Triton
1	2.15	2.55	5.81	7.28	37	35
2	2.34	2.35	5.85	8.7	40	27
3	1.55	1.30	4.84	5.91	32	22
4	1.50	1.20	4.41	6.00	34	20
5	1.25	1.15	3.85	5.23	32	22

**Table 4.1** A summary of results relating to measured amounts of Rubisco and soluble protein in five separate wheat leaf samples, extracted with a buffer in which the detergent Triton-X-100 was either absent (-Triton) or present (+Triton).

However, the presence of Triton-X-100 significantly ( $P=0.051$ ) increased the amount of soluble protein measured in each sample, by an average of 34%. Consequently, since the amount of Rubisco was not significantly affected by this treatment, Rubisco as a proportion of soluble protein decreased.

### *Discussion*

The fact that there was no increase, but if anything a decline in the amount of Rubisco measured when Triton-X-100 was present, suggests that in this wheat cultivar, any Rubisco that may be bound to membranes, is sufficiently removed by extraction buffer 'A'. However, it does not rule out the possibility that the concentration of Triton-X-100 used was insufficient to remove very tightly bound Rubisco which may or may not have still been present, even though the detergent concentration used was at the upper range of that suggested by Makino and Osmond (1991), so making this seem unlikely. Therefore, since the inclusion of

Triton-X-100 had no positive effect on the recovery of Rubisco, and because the presence of this detergent can amplify estimations of soluble protein content (Bradford, 1976) as is apparent here, Triton-X-100 was excluded from the extraction buffers used in the studies reported below.

#### 4.2.4 *Section summary*

Given that the inclusion of Triton-X-100, PVPP and the various protease inhibitors in extraction buffers had no significant effect on the recovery of Rubisco extracted from wheat leaf material, the standard Rubisco extraction buffer used for the remainder of this study contained (unless stated otherwise) 50 mM Bicine (pH 8.0), with 20 mM MgCl<sub>2</sub>, 1 mM EDTA and 50 mM  $\beta$ -Mercaptoethanol.

### 4.3 Quantification of Rubisco by ELISA

#### 4.3.1 *Introduction*

Enzyme-linked immunosorbent assays (ELISAs) have become very popular as a means of quantifying proteins of interest, given their high sensitivity, safety, economy and simple instrument requirements. To quantify an enzyme (antigen) of interest for example, the antigen is first adsorbed to wells in a plate (solid phase), as are, separately purified antigen standards and controls. To these wells a primary antibody (Ab<sub>1</sub>) previously raised against the antigen of interest is added, and after sufficient time, any unbound excess is washed away. A secondary antibody (Ab<sub>2</sub>) is then directed against Ab<sub>1</sub>, with the latter linked to a marker enzyme (usually peroxidase). An appropriate substrate can then be added and enzyme activity (colour development) can be

measured (optical density) and related to the primary antigen concentration by comparison with standards.

Such a method for quantifying Rubisco in tobacco had previously been developed at IACR-Rothamsted (briefly described in Paul and Driscoll, 1997), and for which monoclonal Ab<sub>1</sub> against wheat Rubisco was available.

#### 4.3.2 *Materials and method*

Rubisco from approximately 60 mm<sup>2</sup> of leaf, was extracted in a pestle and mortar on ice, in 1 mL of a 50 mM Bicine buffer (pH 8.0) containing 20 mM MgCl<sub>2</sub>, 1 mM EDTA and 50 mM  $\beta$ -Mercaptoethanol. A further 1 mL of buffer was used to rinse the mortar, this was combined with the first, and sub-samples were removed for the determination of Chl. The homogenate was clarified at 10000 xg (4°C) for three minutes, and the supernatant transferred to a clean microcentrifuge tube, from which sub-samples were taken to determine the soluble protein content. At room temperature (20 to 25°C depending on the day), 5  $\mu$ L of antigen (Rubisco sample or Rubisco standard) was diluted 5000-fold in 25 mL of a carbonate coating buffer (pH 9.6 with HCl) containing 60 mM Na<sub>2</sub>CO<sub>3</sub> and 162 mM NaHCO<sub>3</sub>. Using a 96 well ELISA plate, 200  $\mu$ L of diluted samples were applied to wells in the first column only, followed by 50  $\mu$ L of carbonate coating buffer to all remaining wells of columns two to eight inclusive. Using a multi-pipette, a range of antigen concentrations were made horizontally across the plate, by successively removing 150  $\mu$ L from one column to the next. A range of standards were made in columns nine to eleven, by adding 113  $\mu$ L of diluted standard antigen to the first wells of each column, adding 50  $\mu$ L of carbonate coating buffer to remaining wells, and then sequentially removing 63  $\mu$ L from each

well to the next, working vertically down the plate. Wells in column twelve contained controls of either no antigen, no primary antibody, no secondary antibody or no peroxidase substrate. Plates were incubated for 60 minutes at 37°C on damp tissue paper in a sealed Tupperware box, or alternatively left overnight in a fridge at approximately 4°C.

A ten times stock solution of phosphate buffered saline (10x PBS) had previously been prepared which contained 1.4 M NaCl, 15 mM  $\text{KH}_2\text{PO}_4$ , 120 mM  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$  and 27 mM KCl. Following incubation, plates were washed three times for five minutes each in a freshly made ten fold dilution of this stock which additionally contained 0.05% (v/v) Tween 20, and hereafter is called PBS-T. Plates were then incubated for 60 minutes at 37°C with a blocking buffer (200  $\mu\text{L}$  per well) which consisted of PBS-T additionally containing 45  $\mu\text{M}$  PVP and 0.5g/100 mL of NIDO milk powder. After blocking, plates were washed as before in PBS-T, and incubated for 60 minutes at 37°C, with 100  $\mu\text{L}$  per well of primary rat mono-clonal antibody against Rubisco, which had been diluted 1000-fold in blocking buffer. Plates were washed thrice for a third time as before, and reincubated at 37°C for a further 60 minutes with 100  $\mu\text{L}$  per well of secondary rabbit anti-rat IgG peroxidase conjugate (Sigma A-5795) which had been diluted 5000-fold in blocking buffer. Following a further and final three washes in PBS-T, 100  $\mu\text{L}$  of peroxidase substrate containing 0.1 M sodium acetate (pH 5.8), 0.0006% (v/v)  $\text{H}_2\text{O}_2$  and one TMB tablet dissolved in 100  $\mu\text{L}$  of DMSO per 10 mL, was added per well, and the plate left at room temperature for exactly 20 minutes to allow the reaction and colour development to progress. The reaction was stopped by adding 25  $\mu\text{L}$  of 3M  $\text{H}_2\text{SO}_4$  per well, after which absorbencies were read at  $A_{450}$  nm in an ELISA plate reader. For all control

wells, the mean absorbance value was calculated and subtracted from absorbance values for standards and samples. Mean standard values were plotted, to which a curve was fitted, and the equation used to transform absorbance values for unknown samples into amounts of Rubisco.

Wheat Rubisco standard had already been prepared using a method developed by M.A.J. Parry, IACR-Rothamsted (described in Gutteridge et al., 1982) and was supplied as a 3.8 mg/mL stock, which had previously been assessed to be of a purity >95%.

To test the applicability of this method for this study, a preliminary analysis of the amount of Rubisco in leaves of spring wheat was undertaken. In compost, six plants were grown per 150 mm diameter pot on a glasshouse bench at 18/12°C, receiving ample N and natural irradiance. When the third leaf (L3) was beginning to emerge, 100 mm<sup>2</sup> sections from the middle of six second leaves (L2) and six first leaves (L1) were harvested and pooled per leaf insertion number to make two samples from which Rubisco was extracted and analysed.

Expressed on a leaf area basis, L1 contained a greater amount of all components than L2, presumably reflecting a greater total leaf N content acquired over a longer leaf duration. The absolute values obtained for all components fell within the range of those reported elsewhere in the literature for wheat (Evans, 1983; Delgado et al., 1994), with the ratios between components matching well with those extracted from the combined data of tables one and two in Delgado et al., (1994: Rubisco:chlorophyll ratio 6.9 ( $\pm$  0.28 SE), soluble protein:chlorophyll ratio 11.7 ( $\pm$  0.26 SE), where n = 16). However, for wheat, upper values of 43%  $\pm$  0.85 SE (Wittenbach, 1979), 50% (Evans and Austin, 1986) and 45% (Evans and Seeman, 1989) have previously been found for



Rubisco as a percentage of total soluble protein, and since values obtained in this experiment were 63 and 53% for L1 and L2 respectively (data not shown), this suggested that absolute amounts of Rubisco measured with this ELISA could have been overestimated.

Subsequently the purity of the wheat Rubisco standard (which had been stored as multiple aliquots) was reassessed in the event that it may have deteriorated with long term storage under liquid N<sub>2</sub>. Using quartz cuvettes, a Carey dual-beam spectrophotometer and the following expression and extinction coefficient:

$$\text{Rubisco concentration (mg/mL)} = 280_{\text{Abs}} - 320_{\text{Abs}} \times 0.61 \quad [4.1]$$

the purity of Rubisco standard (the concentration of Rubisco subtracted from the total amount of soluble protein (see section 2.5) in the same sample) reassessed on several different occasions was found to be 92% ( $\pm 2.2$  SE), and on this basis the experimental data was recalculated for which results are shown in Table 4.2.

Leaf	Rubisco g m <sup>-2</sup>	S. Protein g m <sup>-2</sup>	Chl g m <sup>-2</sup>	Rubisco % of SP	Rubisco: Chl	S.Prot: Chl
L1	3.97	6.83	0.64	58	6.21	10.7
L2	2.58	5.25	0.43	49	6.0	12.2

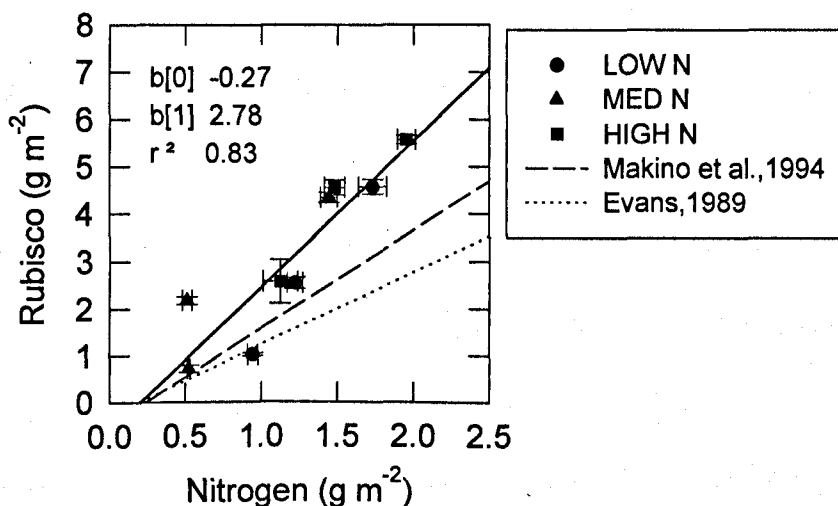
**Table 4.2** Results, after corrections for the purity of Rubisco standard, for the component composition of pooled samples of leaves one (L1) and two (L2) of seedlings of spring wheat grown with ample nitrogen under glasshouse conditions. The amount of Rubisco was estimated by ELISA, soluble protein by the method of Bradford (1976; see section 2.5) and Chl using the 80% acetone method of Arnon (1949; see section 2.4). For further details see text.

Correcting for the standard impurity decreased the absolute amounts of Rubisco by 7.9%, which in turn resulted in Rubisco representing smaller fractions of soluble

protein. This brought values more in line with previous findings in the literature, although they were still at the upper end of reported ranges. At the time, since the plants sampled were in the early stages of growth and had been grown with a sufficient supply of nitrogen, it was considered that the high percentages of soluble protein that Rubisco represented, could be accounted for by these factors.

Subsequently the method was applied to a selection of samples from Experiment One (described in Chapter Two), which had been collected and stored in liquid N<sub>2</sub> following gas exchange analysis, and consisted of flag leaves from full expansion through to senescence, grown at 36 Pa CO<sub>2</sub> and three different N applications. For each sampling occasion and treatment, six leaf samples were taken, for which three were assayed for Rubisco content, and three for total leaf N (described in section 2.7).

The relationship obtained between Rubisco and total leaf N content was linear and independent of N applied during growth (Fig. 4.3), being in accordance with observations for the same relationship in wheat (Evans, 1983; Evans 1989) and rice (Makino et al., 1994; Makino et al., 1997). The positive intercept on the x-axis results in a greater than proportional increase in the fraction of N invested in Rubisco with increasing total leaf N, such that in going from 1 to 2 g N m<sup>-2</sup>, the percentage of total N invested in Rubisco increases from 42 to 47%. A similar response has previously been reported in rice, spinach, pea and bean (Makino et al., 1992), and sometimes for wheat (Evans, 1989) but not always (Evans, 1983; Makino et al., 1992). However, the absolute values for the fraction of N invested in Rubisco reported here (>40%), appear to be approximately double those previously reported for wheat; 20% (Evans and Seeman, 1984; Evans and Austin, 1986), 23% (Evans, 1989) and 28% (Makino et al., 1992), suggesting that the ELISA was still overestimating the amount of Rubisco.



**Figure 4.3** The amount of Rubisco estimated by ELISA versus total leaf N content of flag leaves from full emergence through to senescence, grown at current  $pC_a$  (36 Pa) and N treatments as denoted by the key, and taken from Experiment One. Points are the mean of three N versus the mean of three Rubisco values in leaves of the same occasion and treatment, with 1 x Std Err shown for both axis of each. The line is a first order linear regression, for which the equation is shown. The dashed and dotted lines for the same relationship also in wheat are shown for comparison, and are taken respectively from Makino et al., (1994), and Evans, (1989). For further details see text.

Indeed, addition to Figure 4.3 of lines for the same relationship taken from Makino et al., (1994) and Evans (1989) reveals a large discrepancy, with a decrease of between 36 to 50% being required to bring the absolute Rubisco values estimated by ELISA into line with these previous observations. This overestimation implies that a given amount of Rubisco in the sample is more antigenic than the same amount in the standard. Therefore, based on this finding, the following tests were done, to try and determine whether the standard was less antigenic or conversely the sample was more antigenic than expected, resulting in the apparent overestimation.

#### 4.3.3 Comparison of different Rubisco standards

One possible explanation for the apparent overestimation could be that the primary antibody (Ab<sub>1</sub>) does not bind to all the available epitopes on the Rubisco protein standard. Some epitopes may have become damaged or removed in the

purification process, which involved passage through several different purification columns, or may subsequently have been degraded during storage. Thus, in this test, two leaf samples were quantified against two standard curves on the same ELISA plate; the first utilising the previously used (original) wheat Rubisco standard, and the second using a wheat Rubisco standard (new) which at the time had recently been purified and was a gift from W. Martindale, IACR-Rothamsted. The assay was performed following the standard protocol described in section 4.3.2, with the two standard curves being identical in terms of the amount of standard added (based on measurements of soluble protein content) to comparable wells. Sample one was a flag leaf taken from Experiment One, and sample two a primary leaf, taken from a wheat seedling grown on compost in a tray on a glasshouse bench. Results are summarised below in Table 4.3.

Sample	Standard Used	Rubisco g m <sup>-2</sup>	S. Protein g m <sup>-2</sup>	Chlorophyll g m <sup>-2</sup>	Rubisco % S. Prot	Rubisco: Chlorophyll	S.Protein: Chlorophyll
1	Original	3.04	4.0	0.54	76	5.6	7.4
1	New	3.92	4.0	0.54	98	7.3	7.4
2	Original	3.59	3.9	0.49	92	7.3	8.0
2	New	4.45	3.9	0.49	114	9.1	8.0

**Table 4.3** Comparison between two Rubisco standards ('original', and at the time 'new'), to quantify Rubisco from two wheat leaf samples by ELISA. For further details see text.

It is immediately apparent that the amounts of Rubisco estimated using the new standard were even higher than those assessed using the original, and further, that all estimations appeared inaccurate given that values for Rubisco as a percentage of soluble protein ranged from 76 to 114%. Whilst these results do not eliminate the

possibility that both standards may have lost or damaged epitopes (since they were both prepared by the same method; Gutteridge et al., 1982), it does demonstrate that the original standard was no less reliable, and indeed may have been slightly better than the new. Of interest is the fact that the new standard increased the estimated amount of Rubisco in both samples by approximately 26%, suggesting that the overestimation may be a constant factor for diverse samples.

#### 4.3.4 *Section summary*

In summarising the work described in this section, and the applicability of the ELISA technique for quantifying Rubisco to the level of accuracy required in this study, it appears that the method works well as a relative measure, but less so in absolute terms, where Rubisco is consistently overestimated by almost two-fold.

Although much work has been done with wild-type and transgenic tobacco using immunological techniques to assay Rubisco (Rodermeier et al., 1988; Quick et al., 1991; Hudson et al., 1992), little information is available for this species for the relationship of Rubisco to leaf N. Evans et al., (1994) report from limited data that between 16 to 18% of leaf N was invested in Rubisco of wild-type tobacco, but unfortunately Paul and Driscoll (1997), who report the ELISA method used here, do not provide such data. However, by extracting mean wild-type values for Rubisco and Chl content from Paul and Driscoll, (1997; Figure 3), the average Rubisco:Chl ratio for means, over an eight day period computes to be  $7.45 \pm 0.21$  Std Err, with  $n = 4$ , a ratio almost double the average ratio of 4.44 for Rubisco:Chl in tobacco, extracted from Evans et al., (1994; Table 1). Further, in Evans et al., (1994), tobacco was grown at a PPFD of  $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$ , and in Paul and Driscoll (1997) at a PPFD of  $600 \mu\text{mol m}^{-2} \text{s}^{-1}$ . With a lower growth PPFD, one might expect proportionately more N to be invested

in Chl than Rubisco, so lowering the expected Rubisco:Chl ratio. It seems that the comparatively high Rubisco:Chl ratio for wild-type tobacco plants in Paul and Driscoll (1997), could be explained by an overestimation of the amount of Rubisco, as assessed by the same ELISA technique used in this study on wheat. An alternative method for accurately quantifying Rubisco was sought.

#### 4.4 [ $^{14}\text{C}$ ]CABP binding assay for the quantification of Rubisco

##### 4.4.1 *Introduction*

This method is based on that of Yokota and Canvin (1985) and has been extensively applied (e.g. Delgado et al., 1994), and relies on the binding of  $^{14}\text{C}$  radio-labelled 2-carboxyarabinitol 1,5-bisphosphate ([ $^{14}\text{C}$ ]CABP) to the active sites of Rubisco, CABP being an analogue of an intermediate of the carboxylation/oxygenation reaction of RuBP. Estimates of the total functional holoenzyme in extracts is obtained by fully activating (carbamylating) Rubisco in the presence of sodium hydrogen carbonate and magnesium ions, with sodium sulphate present to remove any tight-binding inhibitors of active sites. Under these *in vitro* conditions and at a physiological pH, [ $^{14}\text{C}$ ]CABP binds to all active sites, so Rubisco is quantified assuming a molar ratio of bound [ $^{14}\text{C}$ ]CABP : Rubisco of 8:1.

##### 4.4.2 *Preparation of [ $^{14}\text{C}$ ]CABP*

Two hundred  $\mu\text{L}$  aliquots of [ $^{14}\text{C}$ ]CABP stored at  $-20^\circ\text{C}$  had previously been prepared and provided by the laboratory of Andralojc, Keys and Parry (IACR-Rothamsted, Harpenden, UK). On the day prior to use, required aliquots were made

up to 1 mL with distilled H<sub>2</sub>O (pH 7.5 with NaOH) and left at room temperature overnight, allowing the [<sup>14</sup>C]CABP to adopt an open linear conformation for the assay.

#### 4.4.3 *Sample preparation*

Unless otherwise stated, the following was done between 0 – 4°C. In a pestle and mortar on ice, individual leaf samples of known area (approximately 600 mm<sup>2</sup>) were ground in 1 mL of extraction buffer (optimised for Rubisco removal), that contained 50 mM Bicine (pH 7.5 with NaOH), 20 mM MgCl<sub>2</sub>, 1mM EDTA and 50 mM  $\beta$ -Mercaptoethanol. The homogenate was transferred to a micro-centrifuge tube, to which a further 1 mL of extraction buffer was added after it had been used to rinse any remaining homogenate from the mortar. The sample was briefly mixed for 5 s, and two 50  $\mu$ L aliquots were taken and kept on ice in the dark for later determination of Chl concentration (see section 2.4). The remainder was clarified twice at 10000 xg for 3 minutes, the supernatant being retained and pellet discarded on each occasion. In this way, six samples were prepared on each day that the assay was done, extracted samples being kept on ice (never for more than 30 minutes) whilst remaining samples were prepared.

#### 4.4.4 [<sup>14</sup>C]CABP Assay

An activating buffer stock solution was prepared, comprising 400 mM Bicine (pH 8.0 with NaOH), 80 mM MgCl<sub>2</sub>, 40 mM NaHCO<sub>3</sub> (added under high pH conditions so as not to liberate the CO<sub>2</sub> required to carbamylate Rubisco) and 200 mM  $\beta$ -Mercaptoethanol. One hundred  $\mu$ L of this buffer, 40  $\mu$ L of 1M Na<sub>2</sub>SO<sub>4</sub>, 35  $\mu$ L of dH<sub>2</sub>O and 25  $\mu$ L of the [<sup>14</sup>C]CABP prepared stock were added and mixed in a 1.5 mL micro-centrifuge tube. To this, 200  $\mu$ L of sample was added, the contents briefly mixed, and allowed to stand on ice for 15 minutes, with occasional re-mixing. To

precipitate protein, 288  $\mu\text{L}$  of 60% PEG was added, the contents of the micro-centrifuge tube shaken briefly, and allowed to stand on ice for a further 30 minutes. Precipitate was pelleted by centrifugation at 10000  $\times g$  for 10 minutes ( $4^{\circ}\text{C}$ ), and the supernatant drawn off and discarded. The pellet was washed in 400  $\mu\text{L}$  of 20% PEG, and left on ice for 15 minutes. Where pellets were large, it was necessary to resuspend them to release any potentially unbound [ $^{14}\text{C}$ ]CABP trapped within. Again the protein was pelleted as before, and the wash step repeated a second time. Lastly, pellets were resuspended in 1 mL of Triton X-100, and left to stand at room temperature overnight. Nine hundred  $\mu\text{L}$  of sample was then placed in a scintillation vial and mixed with 3.5 mL of scintillation cocktail (Ultima-Gold) and radioactivity within the sample was counted in a Packard 2500 TR Liquid Scintillation Analyser.

#### 4.4.5 *Calculations*

Values for the amount of Rubisco in a sample were derived from the fact that 1  $\mu\text{g}$  of Rubisco gave a decay per minute (DPM) value of 32.28 (Dr M.A.J Parry, personal communication). This was for a 200  $\mu\text{L}$  sub-sample of a 2 mL leaf extract. Thus correcting for dilution Rubisco content in the original 2 mL leaf extract, which was expressed on a leaf area basis, was calculated as:

$$\mu\text{g Rubisco in 2 mL extract} = \text{DPM value} / 32.28 \times 1.11 \times 10 \quad [4.2].$$

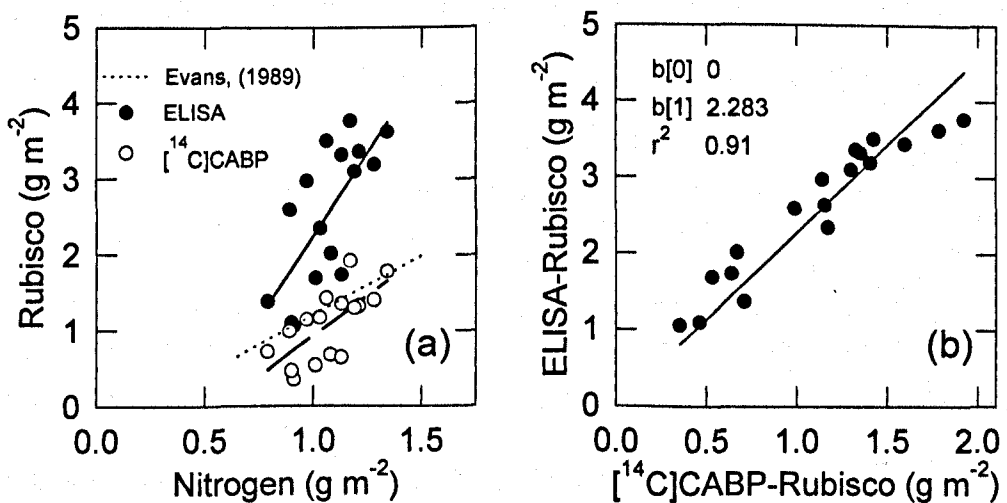
#### 4.4.6 *Preliminary testing of the [ $^{14}\text{C}$ ]CABP binding assay*

To test the reliability and accuracy of the method, samples were assayed from flag-1 and flag leaves selected from Experiment Two (full details in Chapter Two), all of which were taken from plants grown at 36 Pa  $\text{CO}_2$  and the low N application.



Further, at the same time a comparison was made between this and the ELISA method for estimating the amount of Rubisco in the same samples, for which results are shown in Figure 4.4 a.

Regardless of the assay used, the relationship between Rubisco and leaf N content was approximately linear, in both cases positively intercepting the x-axis, which as discussed in section 4.3.2, results in a greater than proportional increase in the fraction of N invested in Rubisco with increasing total leaf N content (Fig. 4.4 a). The ELISA and [ $^{14}\text{C}$ ]CABP regressions, respectively intercepted the x-axis at 0.5 and 0.57  $\text{g m}^{-2}$  N, values which are higher than usual (e.g. 0.25  $\text{g m}^{-2}$ , the line of Evans, (1989) as shown), but which presumably are a reflection of the narrow range of N contents that samples contained, and through which the linear regressions were plotted.



**Figure 4.4 a** The relationship between Rubisco and N content in flag-1 and flag leaves of wheat grown at 36 Pa  $\text{CO}_2$  and harvested on several occasions from full expansion through to senescence, where Rubisco has been measured both by ELISA and the binding of [ $^{14}\text{C}$ ]CABP in the same sample. Both lines represent first order regressions; for ELISA (full line)  $Y = 4.394 X - 2.144$ ,  $r^2 = 0.51$  and for [ $^{14}\text{C}$ ]CABP (dashed line)  $Y = 2.155 X - 1.228$ ,  $r^2 = 0.48$ . **b** Correlation for the amount of Rubisco as determined by the two assay methods. The regression is forced through the origin. For further details see text.

Clearly for the ELISA method, for a given leaf N content the amount of Rubisco is greater than twice that assessed by the [ $^{14}\text{C}$ ]CABP assay, and this supports previous discussion in section 4.3.5, that the ELISA method appeared to be overestimating the amount of Rubisco by approximately two-fold. Indeed, this is confirmed by Figure 4.4 b, in which the Rubisco value obtained by ELISA is plotted against the value obtained for the same extract using the [ $^{14}\text{C}$ ]CABP assay. A correlation close to linearity is obtained, which when the slope is adjusted slightly to pass through the origin, gives a correction factor of 2.28.

Thus, on the basis of these results, the [ $^{14}\text{C}$ ]CABP binding assay became the preferred choice for estimating the amount of Rubisco in samples, a decision further supported by the fact that the slope obtained using the [ $^{14}\text{C}$ ]CABP assay (Fig 4.4 a), agrees reasonably well with the slope for the same relationship in wheat found by Evans, (1989).

The ELISA method had previously been applied to all samples in Experiment One so that based on the above, and the relationship between estimated amounts of Rubisco and corresponding relationships with Chl, soluble protein and various gas exchange parameters (data presented in Chapter Three), all ELISA estimations for Rubisco were corrected by dividing by a factor of 2.28, and any subsequent presentation of amounts of Rubisco determined by ELISA have been corrected in this way, unless stated otherwise.

## 4.5 Measurement of Rubisco initial, total and maximal activities

### 4.5.1 Introduction

In general, it is believed that Rubisco which is in excess of that required to maintain photosynthetic rate is deactivated (Sage et al., 1992), so that the concentration of RuBP in the stroma tends to be constant for variable rates of RuBP-regeneration. The regulation of Rubisco activity in a variable environment is complex and is achieved by a number of different steps. This occurs through changes in the reversible carbamylation of a lysine 201 residue, which additionally requires the binding of  $Mg^{2+}$  to form a catalytically active ternary complex (Miziorko and Lorimer, 1983). Superimposed upon this mechanism are several others including, (1) the light-dependent activation of Rubisco by Rubisco activase, (2) inhibition by 2-carboxy-D-arabinito-1-phosphate (CA1P), and (3) modulation by stromal metabolites such as inorganic phosphate (Parry et al., 1985; Gutteridge et al., 1986; Portis et al., 1986). Whilst (2) is an important mechanism of regulation in species such as tobacco, sugar beet and potato, evidence for the presence of this inhibitor in wheat and barley is lacking (Parry et al., 1997), although there is growing evidence that alternative inhibitors may be present in these latter species (Keys et al., 1993).

'The degree to which Rubisco is carbamylated *in vivo* may be determined by the extent to which its 'initial' activity, measured immediately in extracts prepared rapidly in the cold, is less than the 'total' activity measured following incubation with saturating concentrations of  $CO_2$  and  $Mg^{2+}$  to carbamylate vacant lysine 201 residues in the active site. However, as Parry et al., (1997) discuss, the term total activity is a misnomer since it does not include sites blocked by tightly bound inhibitors, which are not removed by incubation with  $CO_2$  and  $Mg^{2+}$ . This results in the potential

underestimation of the activity of fully activated, non-inhibited Rubisco. Thus, whilst differences between initial and total activities represents a measure of regulation by carbamylation, so changes in total activity represent regulation by CA1P or similar inhibitors. The degree to which the binding of inhibitors decreases total activity, can be determined by measurement of the catalytic activity before and after their removal by incubation of the extract in the presence of  $\text{Na}_2\text{SO}_4$ .

It was of interest in this study, using the method of Parry et al., (1997), to investigate how growth of wheat plants under elevated  $\text{CO}_2$  might alter the degree to which carbamylation and the binding of inhibitors exert control on Rubisco activity. This is of particular importance to this study, since from the arguments above, the percentage of Rubisco which is not active *in vivo* may be taken as a measure of excess Rubisco capacity for the environment in which it was isolated.

#### 4.5.2 *Materials and Method*

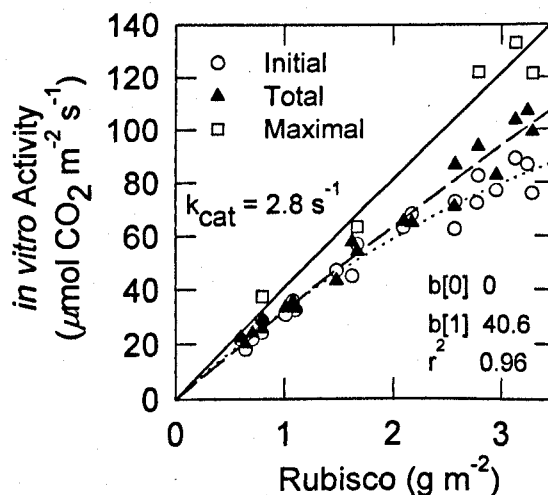
Rubisco from leaves ( $400\text{--}500\text{ mm}^2$ ) freeze clamped under saturating light and at their growth  $pC_a$ , was rapidly extracted in 1 mL of  $\text{CO}_2$ -free buffer containing 50 mM Bicine (pH 8.0), 20 mM  $\text{MgCl}_2$ , 1 mM EDTA and 50 mM  $\beta$ -Mercaptoethanol. Extracts were clarified at 10000 xg for 2 min and the supernatant immediately assayed for Rubisco activity. Initial activity was determined at  $25^\circ\text{C}$ , by adding 20  $\mu\text{L}$  of extract to 980  $\mu\text{L}$  of a  $\text{CO}_2$ -free assay buffer containing 100 mM Bicine (pH 8.2) and 20 mM  $\text{MgCl}_2$ , to which immediately prior to the extract,  $[^{14}\text{C}]\text{NaHCO}_3$  (4.6 kBq  $\mu\text{mol}^{-1}$ ) and RuBP had been added to 10 and 0.4 mM respectively. Total activity was determined at  $25^\circ\text{C}$  by incubating 20  $\mu\text{L}$  of extract for 3 min in 980  $\mu\text{L}$  of the same assay buffer, but for the omission of RuBP, and presence of free  $\text{CO}_2$  to allow for the carbamylation of all available active sites. The assay was started by adding

RuBP to 0.4 mM as before. For the determination of maximal activity, 100  $\mu\text{L}$  of extract was incubated for 30 min in an equal volume of 400 mM  $\text{Na}_2\text{SO}_4$  at 4°C, to remove any tight-binding inhibitors that may have been present. This was followed by spin desalting (Helmerhorst and Stokes, 1980) at 400 xg for 2 min at 4°C using Sephadex G25-medium (Pharmacia, Brussels, Belgium), equilibrated with extraction buffer in a small polystyrene column (Pierce, Rockford USA). Determination of maximal activity then followed the procedure for total activity, beginning with the 3 min incubation in the absence of RuBP. All assays were stopped after 1 min by adding 100  $\mu\text{L}$  of 10 M formic acid to liberate any unfixed  $^{14}\text{CO}_2$ , and thereafter evaporated to dryness. Determination of  $^{14}\text{C}$  present was by liquid scintillation spectrometry (model 2500 TR liquid-scintillation analyser, Packard Instruments, Meriden, CT).

#### 4.5.3 *Preliminary assessment*

Figure 4.5 below, shows the results from the application of this assay to wheat flag leaf material taken just as it reached full expansion, for plants grown at a range of N applications (1.8 to 26.3 g m<sup>-2</sup>) and all at current  $pC_a$  (36 Pa) (see section 2.8 for details). Following gas exchange analysis, leaves were freeze clamped under a saturating PPFD of 1500  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$  and at the growth  $pC_a$ , prior to sample storage in liquid N<sub>2</sub>. For each leaf sample, half the supernatant was assessed for Rubisco activity using the method described above, and half used to determine the amount of Rubisco present using the [ $^{14}\text{C}$ ]CABP binding assay described in 4.4.4. Due to the additional steps involved in this preliminary assessment of the method, only five samples were analysed for maximal activity.

The initial and total activities (Fig 4.5) show curvilinear relationships with the amount of Rubisco, whilst the maximal activity is proportional giving an estimated  $k_{\text{cat}}$  of  $2.8 \text{ s}^{-1}$  at  $25^\circ\text{C}$ , and all responses are independent of N applied during growth (actual N treatments not shown). At low Rubisco contents (from 0 to  $1.5 \text{ g m}^{-2}$ ) initial and total activities are identical, but thereafter and with increasing Rubisco content, the initial and total activity responses diverge, such that at a Rubisco content of  $3.0 \text{ g m}^{-2}$ , the initial activity is 15% lower than the total. In absolute terms, the activity values obtained seem reasonable compared to the results of others (e.g. for wheat, Delgado et al., unpublished), with the  $k_{\text{cat}}$  being close to a value of  $3.1 \text{ s}^{-1}$  at  $25^\circ\text{C}$  in wheat estimated by Makino et al., (1988).



**Figure 4.5** Estimates of the *in vitro* Rubisco activity verses Rubisco content in flag leaves of wheat at full emergence, grown at  $36 \text{ Pa CO}_2$  and a range of nutrient treatments to obtain the variation in Rubisco content. Initial, total and maximal activities as denoted by the key, were determined at  $25^\circ\text{C}$  on Rubisco isolated from leaves freeze-clamped under a PPFD of  $1500 \mu\text{mol quanta m}^{-2} \text{ s}^{-1}$  and at the growth  $p\text{CO}_2$ . Lines are second order linear regressions forced through the origin, except for maximal activity where a first order linear regression was used, and for which the equation is shown. For further details see text.

However, the apparent decrease in activation state (initial activity/maximal activity) with increasing Rubisco content, for example down to 65% at a Rubisco

content of  $3.0 \text{ g m}^{-2}$ , is unexpected since it has been assumed that all Rubisco is utilised at high light and the currently limiting  $pC_a$  (36 Pa) for which there is good experimental evidence (Makino et al., 1988; Sage, 1990). Part of the decrease in activation is associated with an inhibitor, demonstrated by the difference between total and maximal activity (Parry et al., 1997). Initial activity is known to be affected by the pre-treatment of the extraction buffer and time taken to determine activity (Sage, 1993), which may have lowered absolute measurements of initial activity here. However, if this is the case, it appears that the deactivation is occurring faster in samples with high Rubisco contents, since the apparent activation state is lower in these samples. Unfortunately, this discrepancy between the expectation of full activation and observed activation state is not easily resolved.

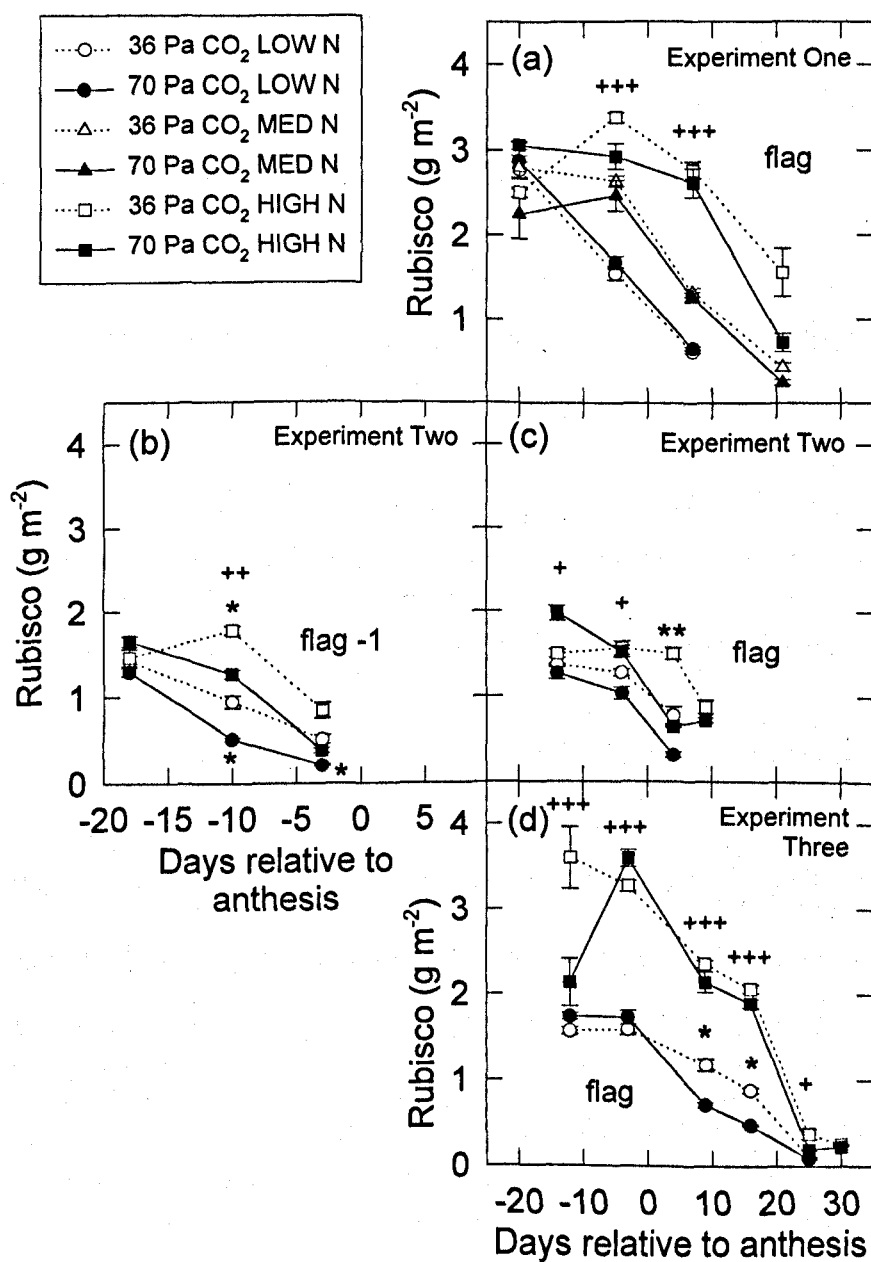
## 4.6 The effect of elevated partial pressures of CO<sub>2</sub> on the amounts of leaf components

### 4.6.1 *Introduction*

To determine whether or not long-term growth at elevated CO<sub>2</sub> would affect the amount of Rubisco in leaves of spring wheat, the techniques discussed above were applied to samples collected from leaves of various insertion, from four separate experiments on plants grown as crop stands, although activities of Rubisco were only measured in the last. The details of these experiments are described fully in Chapter Two, however briefly, for all, plants were grown with total N applications of approximately 8 (low), 15 (medium) or 21 (high) g N m<sup>-2</sup>, and in Experiments One to Three, at either 36 or 70 Pa CO<sub>2</sub> and in Experiment Four at 36 or 100 Pa CO<sub>2</sub>. From tillering to maturity plants were grown with day/night temperature regimes of 20/7°C, and a constant artificial PPFD of 580  $\mu$ mol quanta m<sup>-2</sup> s<sup>-1</sup> at plant height. Experiment One was additionally supplemented with natural radiation. Leaf samples for analysis (which correspond to those also used for gas exchange analysis as discussed in Chapter Three), were harvested from full leaf emergence, through grain-filling and into senescence, at approximately ten day intervals. Results are presented and discussed, first as a function of leaf area through time, and then as a function of total leaf N.



#### 4.6.2 Results - Amount of Rubisco in leaves as a function of time



**Figure 4.6** The response through time, as days relative to anthesis, of the amount of Rubisco measured in flag-1 (b) and flag leaves (a, c, d) of wheat from experiments One (a), Two (b and c) and Three (d), grown under regimes indicated by the key. Each point represents the mean of three samples, and where visible error bars = 1 x Std Err. Asterisks denote levels of significance from CO<sub>2</sub> ANOVA within the N treatments to which they are closest, where \* =  $P < 0.05$  and \*\* =  $P < 0.01$ . Plus signs denote levels of significance from N ANOVA on the occasion indicated, where + =  $P < 0.05$ , ++ =  $P < 0.01$  and +++ =  $P < 0.001$ . In Experiment One Rubisco was quantified by ELISA, and in other experiments by the [<sup>14</sup>C]CABP binding assay. For further details see text.

With regard to leaf Rubisco content through time, in all the leaves studied of all experiments, absolute values for Rubisco within a sampling occasion were always greater in plants grown at higher N applications (as clearly illustrated in Fig. 4.6 a & d), so that Rubisco was completely lost first from leaves grown at low N, then medium N and finally high N applications. However, with the exception of Experiment Three, leaf Rubisco contents between different N treatments were not always significantly different. At full leaf expansion (occasion one) in the flag leaf of Experiment One, and flag-1 leaf of Experiment Two (Fig. 4.6 a & b respectively) for example, leaves had similar Rubisco contents regardless of N application treatment, although absolute values between the two experiments were different. There was no effect of growth CO<sub>2</sub> treatment on the amount of Rubisco, in any leaf of any N treatment or experiment, at full leaf expansion (Fig. 4.6).

For flag-1 of Experiment Two (Fig. 4.6 b), CO<sub>2</sub> had a significant effect at 10 and 3 days prior to anthesis on the amount of Rubisco in the low N treatment plants, and in the high N grown plants at 10 days prior to anthesis only. For the flag leaves of all three experiments (Fig 4.6 a c & d) CO<sub>2</sub> had no effect on the amount of Rubisco in leaves prior to 6 days after anthesis, and then subsequently only significantly affected the third occasion in Experiment Two (high N treatment), and the third and fourth occasions in Experiment Three (low N treatment). In all instances where the CO<sub>2</sub> treatment did have an effect within N treatments, the amount of Rubisco was always lower in 70 Pa CO<sub>2</sub> grown plants. There was no significant interaction between N and CO<sub>2</sub>, in any leaf of any N treatment occasion or experiment.

### 4.6.3 Discussion

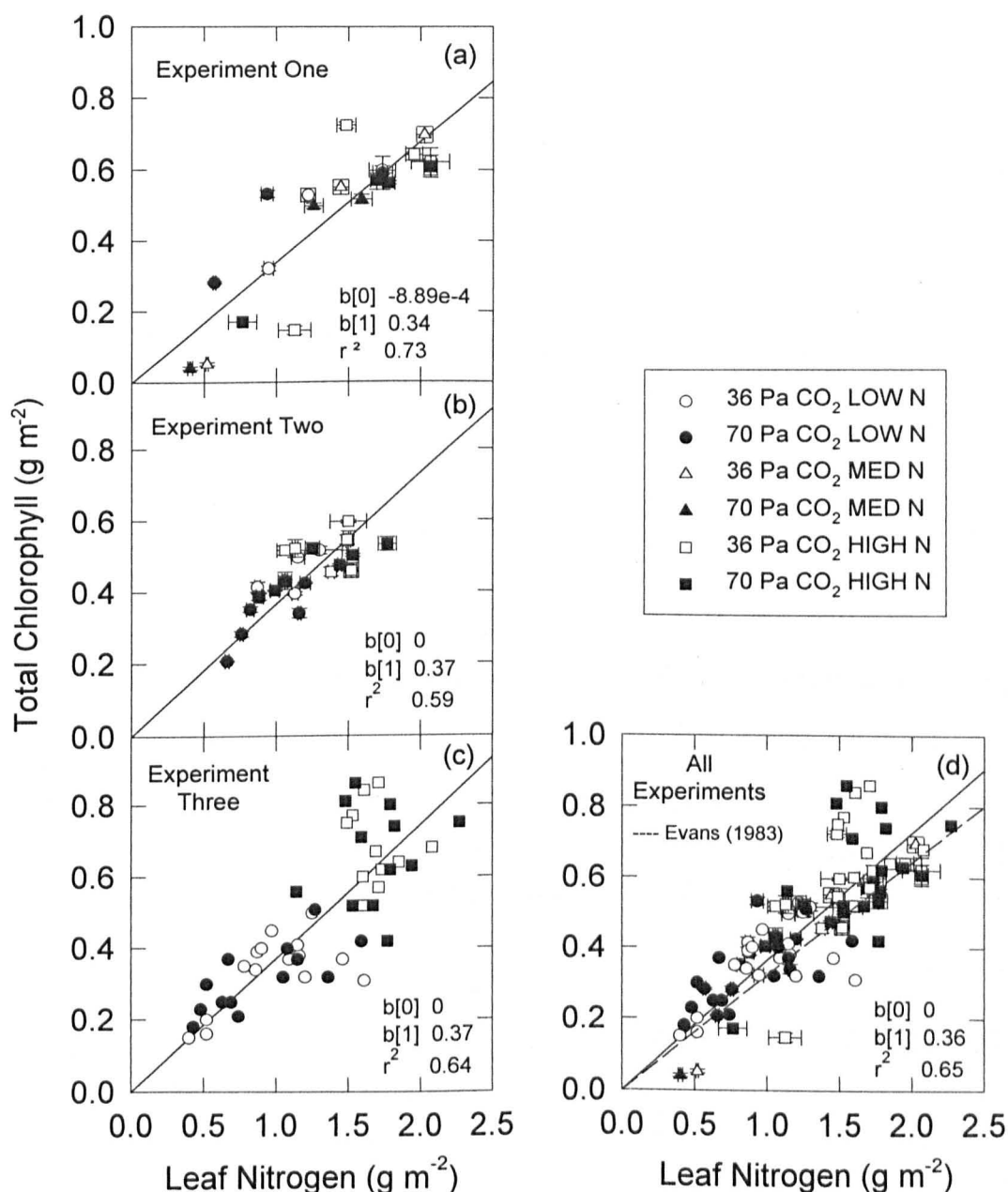
The general decline in absolute values for leaf Rubisco content against time, and regardless of specific treatments, mirror the response of leaf N content against time previously presented in Figure 3.3, and these patterns reflect a natural ontogenic decline within leaves as components are remobilised for the developing ear and grain.

Where there was a significant effect of CO<sub>2</sub> on the amount of Rubisco, absolute amounts were always lower in leaves grown at 70 Pa CO<sub>2</sub>, and this fits with the expectation of the hypothesis being tested, that due to increased Rubisco efficiency, less is required as CO<sub>2</sub> concentration increases. However, as discussed in Chapter One, this observation does not distinguish between reallocation from leaves to sinks, and reallocation from Rubisco to other photosynthetic components. Further, no effect of CO<sub>2</sub> on the amount of Rubisco was seen in any leaf before 6 days after anthesis in any experiment. A similar pattern of response for the amount of Rubisco to elevated CO<sub>2</sub> has been reported for wheat, from the free-air CO<sub>2</sub> enrichment (FACE) experiments of Nie et al., (1995a). Given that the N allocation patterns in developing leaves are likely to be predisposed for 36 Pa CO<sub>2</sub> conditions in which parent plants more recently evolved, this lack of any response prior to 6 days after anthesis, could hypothetically represent the time taken for a mechanism to sense the elevated CO<sub>2</sub> environment in which the leaf has developed, and induce a signal response, which would cause the amount of Rubisco to decline. One such mechanism (discussed in 1.5.3.1) that has been hypothesised, involves a response to carbohydrate accumulation in leaves (van Oosten and Besford, 1996), which feeds back and suppresses gene expression so that the amount of photosynthetic machinery is decreased, to bring source production back in line with sink utilisation. Given that Rubisco has an estimated half-life between 4-7 days, this whole process would take some time to

occur. Alternatively, such a response may only occur, when the leaf becomes a net source of assimilate, and other growing organs put increased demands on the remobilisation of resources contained within it.

Thus, based solely on the observations of Figure 4.6, it seems clear in line with other studies (Peet et al., 1986; Sage et al., 1989; Rowland-Bamford et al., 1991; Jacobs et al., 1995), that long-term growth at elevated CO<sub>2</sub> does have a direct effect on the amount of Rubisco in leaves. However, this could be simply a reflection of lower leaf N contents (Fig. 3.3 of Chapter Three). Therefore, in order to investigate the specific hypothesis of a reallocation from Rubisco to other photosynthetic components (Makino, 1994; Webber et al., 1994; Medlyn, 1996), it is necessary to look for a specific decrease in Rubisco for a given leaf N content. Although in general the pattern of effects on Rubisco (Fig. 4.6) are mirrored by effects on leaf N (Fig. 3.3), since the points plotted in these graphs are mean values, it may be that individually plotted values for Rubisco as a fraction of leaf N measured in the same samples do vary with growth CO<sub>2</sub> treatment, and this is investigated in the next section.

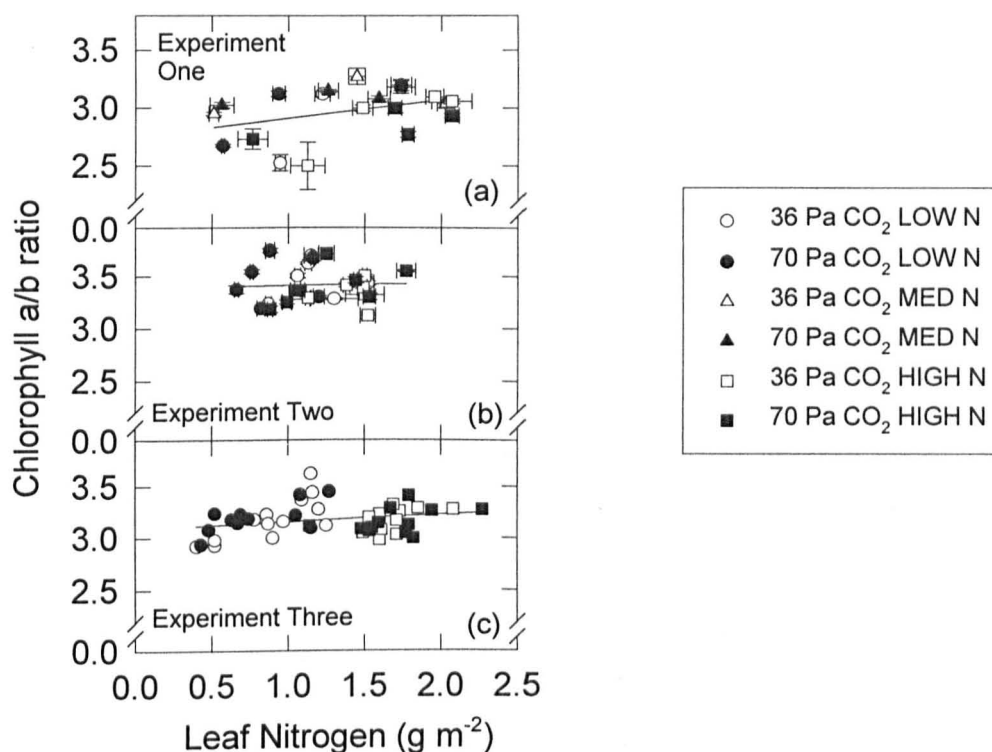
#### 4.6.4 Results - amounts of Chlorophyll, soluble protein and Rubisco as a proportion of total leaf N



**Figure 4.7** Chlorophyll versus total leaf N content measured in samples of flag leaves (Experiments One and Three) or a combination of flag and flag-1 (Experiment Two), from full emergence through to senescence. Plants were grown under the regimes indicated by the key. Points for Experiments One and Two are the mean of three Chlorophyll versus the mean of three N values in leaves of the same occasion and treatment, with 1 x Std Err shown for both axis of each. All panels show first order linear regressions forced through the origin, with the resultant equation. The 'All Experiments' panel shows the combined data for all experiments shown. For further details see text.

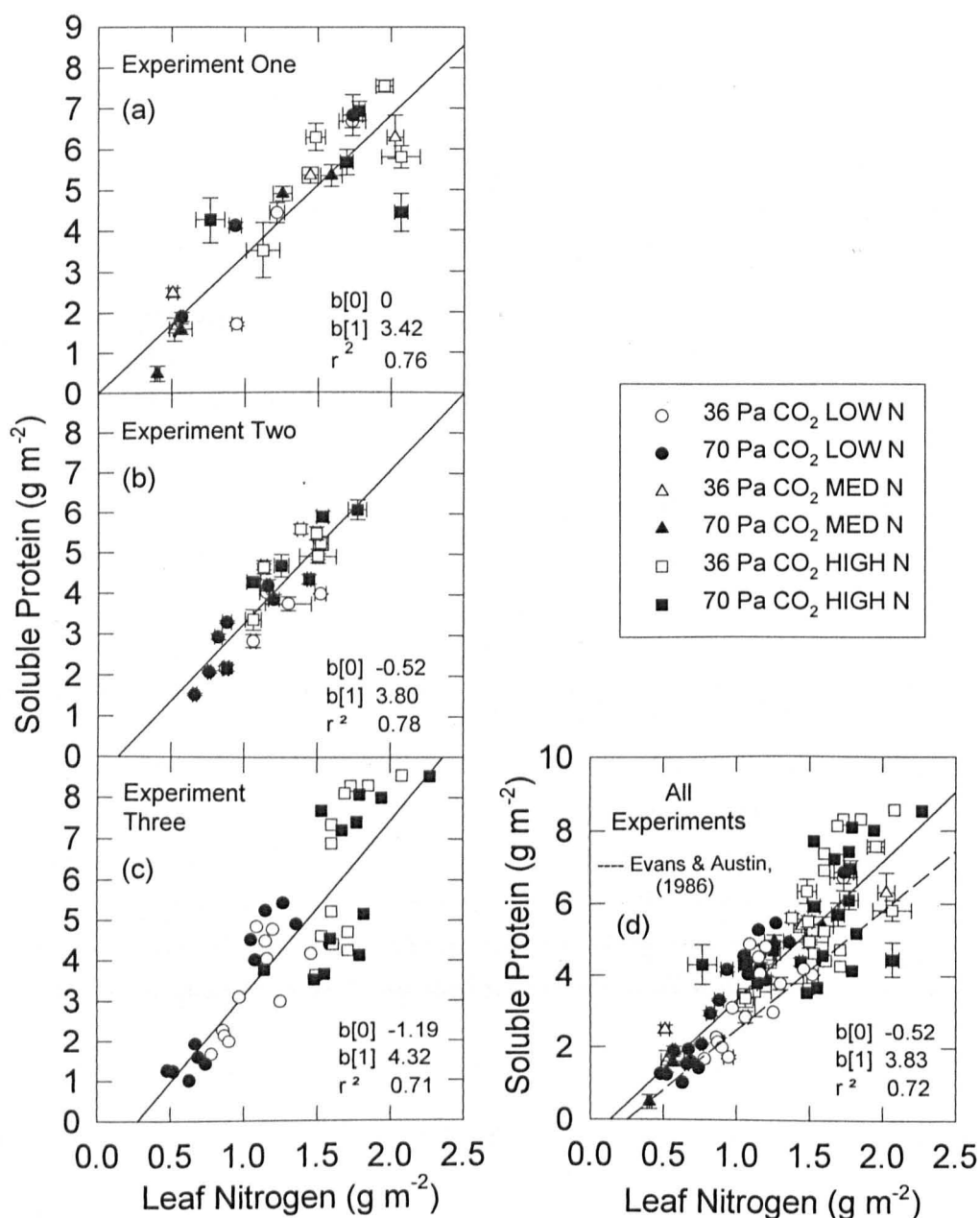
Chlorophyll (Fig. 4.7) was approximately proportional to leaf N content, and the relationship was independent of N and CO<sub>2</sub> treatment, this pattern being consistently seen across all three experiments. Without being forced, regressions for Experiments One and Three (Fig. 4.7 a & c) intercepted the x-axis very close to the origin, whilst the regression for Experiment Two intercepted the y-axis at approximately 0.2 g m<sup>-2</sup> Chl (Fig. 4.7 b). This presumably was a reflection of the narrower range of N contents within leaves that were sampled for this experiment. When data from all three experiments were combined (Fig. 4.7 d), the relationship obtained was very similar to the same relationship for Chl versus N in wheat previously obtained by Evans (1983). Important to note is that because the relationship between Chl and leaf N content are almost proportional, as leaves age, Chl will decline in parallel with leaf N.

The Chlorophyll a/b ratio expressed as a function of leaf N was essentially constant in all three experiments (Fig. 4.8), and again the linear relationship was independent of growth N or CO<sub>2</sub>. The absolute average ratio was lower in Experiment One (Fig. 4.8 a), than in latter experiments.



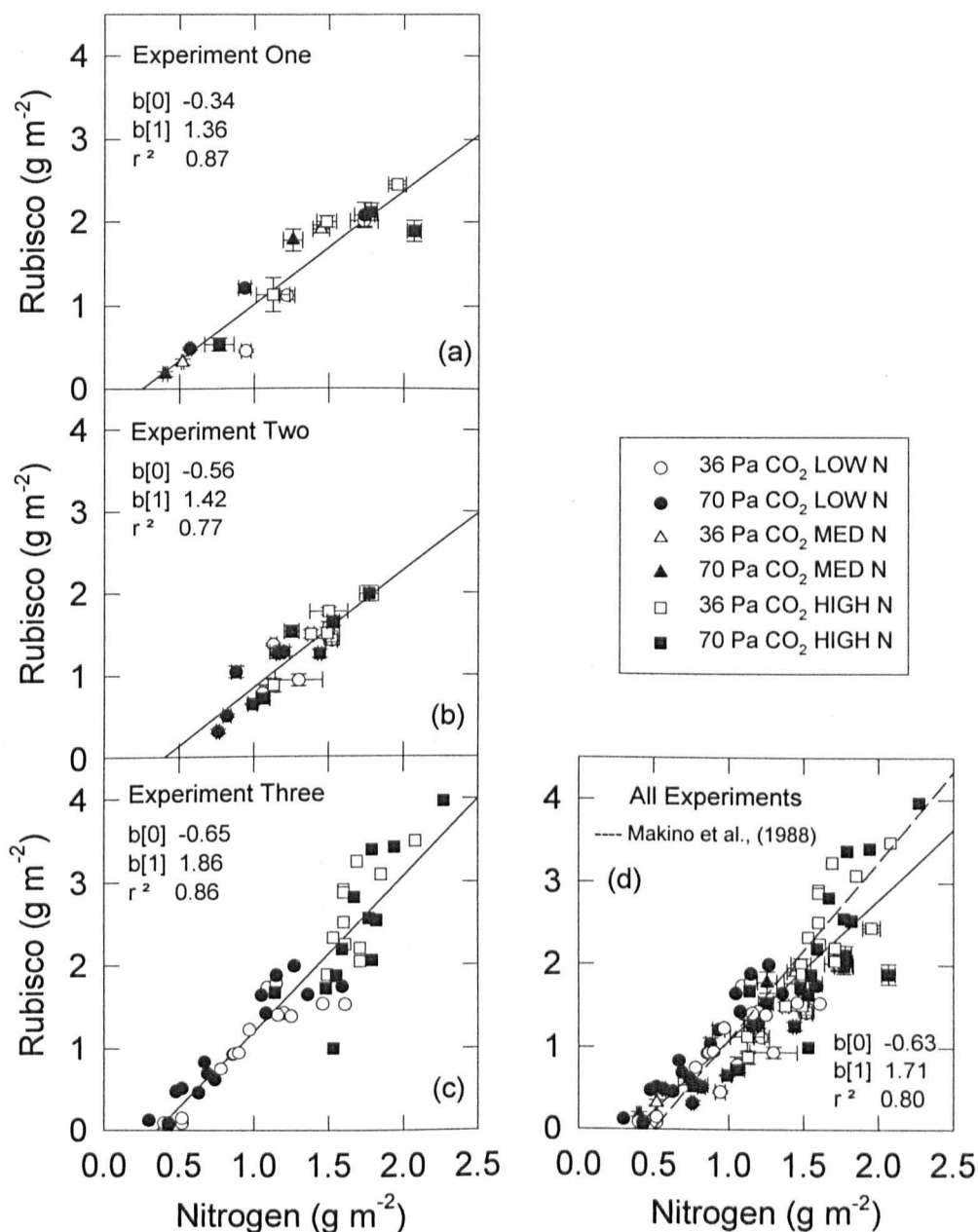
**Figure 4.8** The Chlorophyll a/b ratio versus total leaf N content measured in samples of flag leaves (Experiments One and Three) or a combination of flag and flag-1 (Experiment Two), from full emergence through to senescence. Plants were grown under the regimes indicated by the key. Points for Experiments One and Two are the mean of three Chlorophyll a/b ratios versus the mean of three N values in leaves of the same occasion and treatment, with 1 x Std Err shown for both axis of each. All panels show first order linear regressions. For further details see text.

The relationship between leaf soluble protein and leaf N content was linear (Fig. 4.9), and as for chlorophyll, the relationship was independent of growth N and CO<sub>2</sub> treatment. On average, for the combined data of all three experiments (Fig. 4.9 d), the slope intercepted the x-axis at 0.2 g N m<sup>-2</sup>, suggesting that as leaves age and N is remobilised, soluble protein will decline proportionately faster than leaf N content. Also, the relationship obtained agreed well with the same relationship previously obtained in wheat by Evans and Austin (1986: Fig. 4.9 d).



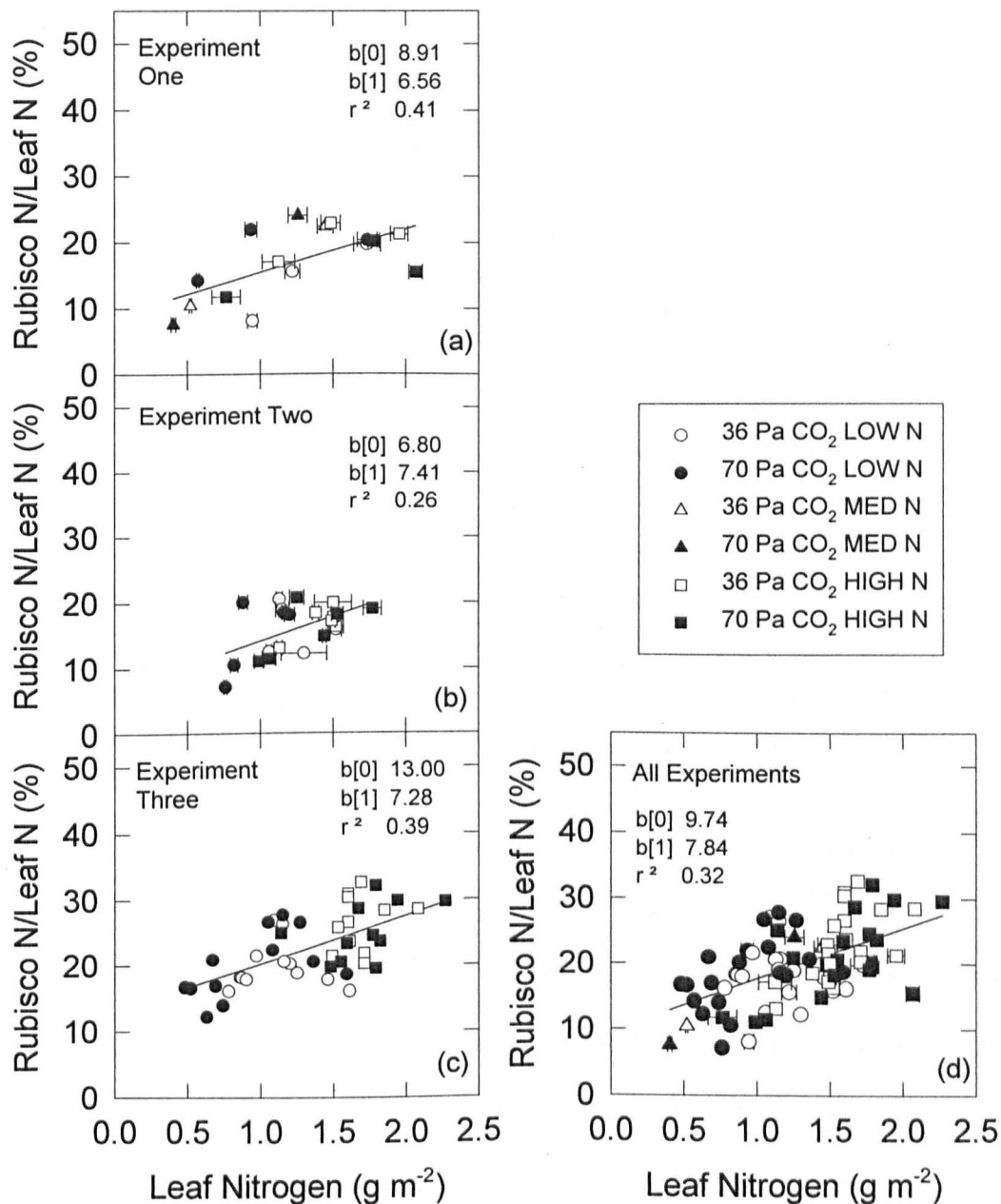
**Figure 4.9** Estimations of soluble protein versus total leaf N content measured in samples of flag leaves (Experiments One and Three) or a combination of flag and flag-1 (Experiment Two), from full emergence through to senescence. Plants were grown under the regimes indicated by the key. Points for Experiments One and Two are the mean of three soluble protein versus the mean of three N values in leaves of the same occasion and treatment, with 1 x Std Err shown for both axis of each. All panels show first order linear regressions, with the resultant equation. Experiment One had a very slight positive intercept on the y-axis, for which reason this regression was forced through the origin. The 'All Experiments' panel shows the combined data for all experiments shown. For further details see text.





**Figure 4.10** Amount of Rubisco versus total leaf N content measured in samples of flag leaves (Experiments One and Three) or a combination of flag and flag-1 (Experiment Two), from full emergence through to senescence. For all experiments, plants were grown under regimes as denoted by the key. Points for Experiments One and Two are the mean of three Rubisco versus the mean of three N values in leaves of the same occasion and treatment, with 1 x Std Err shown for both axis of each. All panels show first order linear regressions and the resultant equation. Rubisco content in Experiment One was estimated using the ELISA technique, and in Experiments Two and Three with the [<sup>14</sup>C]CABP binding assay. The 'All Experiments' panel shows the combined data for all experiments shown. For further details see text.

For all three experiments, the relationship between the amount of Rubisco and total leaf N content was approximately linear and independent of both N application treatment during growth, sampling occasion and CO<sub>2</sub> (Fig. 4.10 a-c). For all, there was a positive intercept on the x-axis, which ranged from 0.15 to 0.4 g m<sup>-2</sup>, suggesting that the fraction of leaf N allocated to Rubisco increases as total leaf N content also increases. This implies that during leaf senescence Rubisco declines proportionately faster than leaf N as a whole. The important conclusion that the Rubisco to leaf N relationship is unaffected by treatments including elevated CO<sub>2</sub>, is perhaps made clearer by presenting the data in the form in Figure 4.11 (a-d), which shows the changes in the proportion of N allocated to Rubisco, which increases with increasing total leaf N content. Again this relationship was linear, and independent of N and CO<sub>2</sub> treatment, and was seen in the leaves analysed from all three experiments.



**Figure 4.11** Rubisco N as a percentage of total leaf N versus total leaf N content measured in samples of flag leaves (Experiments One and Three) or a combination of flag and flag-1 (Experiment Two), from full emergence through to senescence. Plants were grown under the regimes indicated by the key. Each point for Experiments One and Two is the fraction of Rubisco N of total leaf N (where the Rubisco value used was the mean of three, derived from Fig. 4.10) versus the mean of three N values in leaves of the same occasion and treatment, with 1 x Std Err shown for the x-axis. All panels show first order linear regressions and the resultant equation. The original Rubisco estimations from which points in these panels are derived, were estimated using the ELISA technique in Experiment One, and the [<sup>14</sup>C]CABP binding assay in Experiments Two and Three. The 'All Experiments' panel shows the combined data for all experiments shown. For further details see text.

For example, in Experiment Three, the percentage of leaf N allocated to Rubisco, increased from 16 to 31%, with an increase in leaf N content from 0.5 to 2.5 g m<sup>-2</sup>.

#### 4.6.5 Discussion

The relationship between Chl and leaf N content (Fig. 4.7) is linear and independent of CO<sub>2</sub> and N treatments, and corresponds well with observations of the same relationship in wheat (Evans, 1983; 1987) and in rice grown at 36 and 100 Pa CO<sub>2</sub> (Nakano et al., 1997). Importantly the relationship is almost proportional to leaf N content, and this means that as leaves senesce Chl will decline in parallel with leaf N content. It is important to note that Chl is a measure of light harvesting capacity, rather than the other capacities which could limit at high light: namely RuBP-regeneration and PO<sub>4</sub><sup>3-</sup>-regeneration. It is therefore necessary to have a direct measure of a component directly associated with RuBP-regeneration capacity: i.e. the ATP-synthase as discussed and presented in the next chapter.

The relationships between Chl a/b and soluble protein versus leaf N content (Figs 4.8 and 4.9 respectively) were also independent of CO<sub>2</sub> and N application, and were consistently so in the three experiments. The Chl a/b ratio for Experiment One, was on average less than 3.0 compared to averages greater than 3.2 in other experiments. This presumably reflected the exposure of plants in Experiment One to natural irradiance, which was absent in the other experiments. Like the relationship of Chl to leaf N content, soluble protein to leaf N content had a linear relationship, also independent of N and CO<sub>2</sub> treatment, which corresponded reasonably well with that previously reported for wheat (Evans and Austin, 1986).

According to Sage et al., (1989), following a decrease in initial Rubisco activity and activation under elevated CO<sub>2</sub>, if an ideal biochemical acclimation to elevated CO<sub>2</sub> occurs, Rubisco activation should recover, as the amount of excess enzyme as a proportion of leaf N content, is degraded. The data presented in Figure 4.6 shows that on a leaf area basis through time, there was a significantly greater loss of Rubisco in 70 Pa CO<sub>2</sub> grown leaves of various N treatments, but only on occasions at least six days after anthesis. However, the response of leaf N content through time (Fig. 3.3) closely mirrors the response of Rubisco, such that the relationship between Rubisco and leaf N may remain constant. That this is the case, is confirmed by the data presented in Figure 4.10, in which the relationship between leaf Rubisco and leaf N content is demonstrated to be linear and independent of N or CO<sub>2</sub> treatment. The reliability of this observation is supported by the fact that it was consistently observed through the course of three separate experiments, and that when the data from all three is combined (Fig. 4.10 d), the relationship is almost identical for the same relationship in wheat reported elsewhere (Makino et al., 1988; Makino, 1994), although in only one of these experiments (Makino, 1994) was CO<sub>2</sub> a treatment during growth. However, a similar linear response and one independent of N or CO<sub>2</sub> treatment has recently been confirmed for rice (Nakano et al., 1997), although in this latter experiment and that of Makino (1994), leaves were only sampled once at full leaf expansion. It is not unreasonable to hypothesise that an ideal biochemical acclimation to elevated CO<sub>2</sub> might develop later in leaf ontogeny, based on for example the FACE experimental work (Nie et al., 1995a). However, there appears to be little evidence for this, given that relationships presented here, include samples taken throughout leaf development from full expansion through to senescence. Thus, one may conclude that there is no evidence for decreased Rubisco content as a proportion of leaf N under elevated CO<sub>2</sub>,

suggesting that an ideal biochemical acclimation did not take place in these experiments, and that therefore, Rubisco in excess of that required for carboxylation must have persisted for much of the time.

One important aspect of the Rubisco to N relationship (Fig. 4.10) is the positive intercept on the x-axis, which determines that Rubisco does not decline in proportion to leaf N, and so results in an increase in the proportion of N allocated to Rubisco with increasing leaf N content (Fig. 4.11). Again this is a relationship which is independent of CO<sub>2</sub> and N application, and one which corresponds closely to the observations of others (Makino et al., 1997). Using the mean data summarising all three experiments for each of Chl, soluble protein and Rubisco versus N content, the positions of x-axis intercepts, are ordered such that with declining leaf N content, Rubisco, soluble protein and then Chl will be preferentially lost from leaves.

## 4.7 The effect of elevated CO<sub>2</sub> on the activity of Rubisco

### 4.7.1 *Introduction*

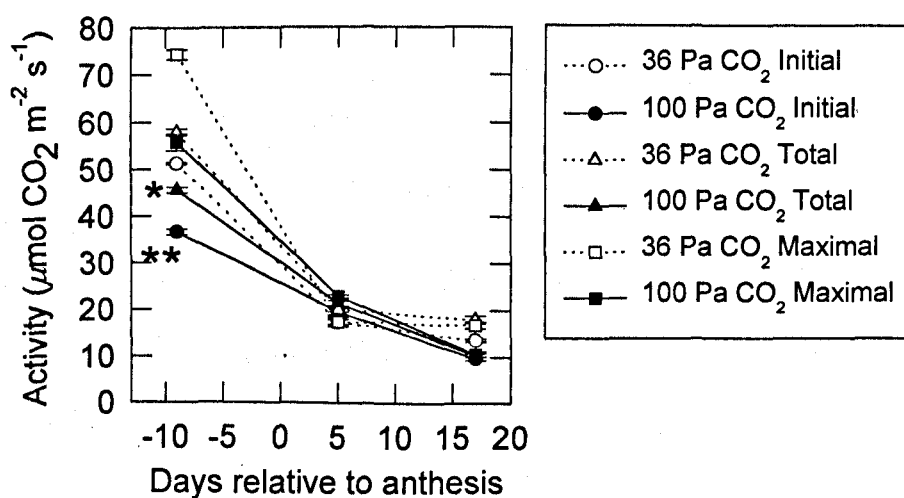
Given the lack of evidence in the preceding section for an optimisation of N within the photosynthetic machinery of plants grown at elevated CO<sub>2</sub> in terms of a specific decrease in Rubisco for a given leaf N content, one can hypothesise that if at elevated CO<sub>2</sub> the balance of capacities are nevertheless maintained and RuBP-regeneration capacity is the most limiting, then there should be a compensatory decrease in Rubisco activation. To determine whether or not this is the case, the technique for determining Rubisco activity described in section 4.5 was applied to samples collected from leaves of various insertion, from Experiment Four. The details of this experiment are described fully in Chapter Two, however briefly plants were

grown with a total N application of  $7 \text{ g N m}^{-2}$ , and at 36 or 100 Pa  $\text{CO}_2$ . A more extreme  $\text{CO}_2$  elevation was chosen to ensure significant effects on activation. From tillering to maturity plants were grown with day/night temperature regimes of  $20/7^\circ\text{C}$ , and a constant artificial PPFD of  $580 \mu\text{mol quanta m}^{-2} \text{ s}^{-1}$  at plant height. Leaf samples for analysis (which correspond to those also used for gas exchange analysis and are discussed in Chapter Three), were harvested from full leaf emergence, through grain-filling and into senescence, at approximately ten day intervals. Results are presented and discussed, first as a function of leaf area through time, and then as a function of total leaf N.

#### 4.7.2 Results-Activity of Rubisco as a function of time

Regardless of treatment or the type of activity (i.e. initial, total or maximal) measured, when expressed on a leaf area basis, all activities measured *in vitro* declined with increasing leaf age (Fig. 4.12 below). Generally, initial, total and maximal activities were always lower in 100 Pa  $\text{CO}_2$  grown leaves, than in their 36 Pa  $\text{CO}_2$  grown counterparts, although significant effects of  $\text{CO}_2$  treatment were only observed for initial and total activities (denoted by asterisks in Fig. 4.12) on the first occasion at 10 d prior to anthesis. On this first occasion, the percentages of activation (initial activity/maximal activity  $\times 100$ ) were approximately 70 and 65% respectively for the 36 and 100 Pa  $\text{CO}_2$  treatments. The difference between total and maximal activity, represents the degree of binding of inhibitors to active sites (Parry et al., 1997), and the data here indicates that there is a significant degree of inhibition in wheat, in that approximately 23 and 20% of potential Rubisco activity in 36 and 100 Pa  $\text{CO}_2$  samples

respectively, was lost at 10 d prior to anthesis, as shown by the difference between total and maximal activities.



**Figure 4.12** The response through time, as days relative to anthesis, of the *in vitro* initial, total and maximal activities of Rubisco (circles, triangles and squares respectively), measured in flag leaves of wheat (Experiment Four), grown at 36 (open symbols) or 100 (closed symbols) Pa CO<sub>2</sub>, and all at low N application. All activities were measured at 25°C on Rubisco isolated from leaves freeze clamped under a PPFD of 1500  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$  and at the growth  $pC_a$ . Each point represents the mean of three samples, and where visible error bars = 1 x Std Err. Asterisks denote levels of significance from CO<sub>2</sub> ANOVA, where \* =  $P < 0.05$  and \*\* =  $P < 0.01$ , and are placed next to the symbol for the type of activity, within which the CO<sub>2</sub> effect occurred. For further details see text.

After anthesis, the percentage activation in samples of both CO<sub>2</sub> treatments increased, and was always greater than 85%, whilst the difference between total and maximal activities decreased to less than 7%.

Since Rubisco measured for maximal activity should be free of any active site inhibitors and fully carbamylated, this measure may be considered to reflect Rubisco amount. On this basis the amount of Rubisco was 22% lower in leaves grown at 100 Pa CO<sub>2</sub> when measured 10 d prior to anthesis, and for the same treatment, 36% lower at 17 d after anthesis. However, at 5 d after anthesis, the amount of Rubisco in 100 Pa CO<sub>2</sub> grown leaves was in fact 27% higher than the average amount of Rubisco in 36 Pa CO<sub>2</sub> grown sample. This observation though, is not likely to be a true reflection of

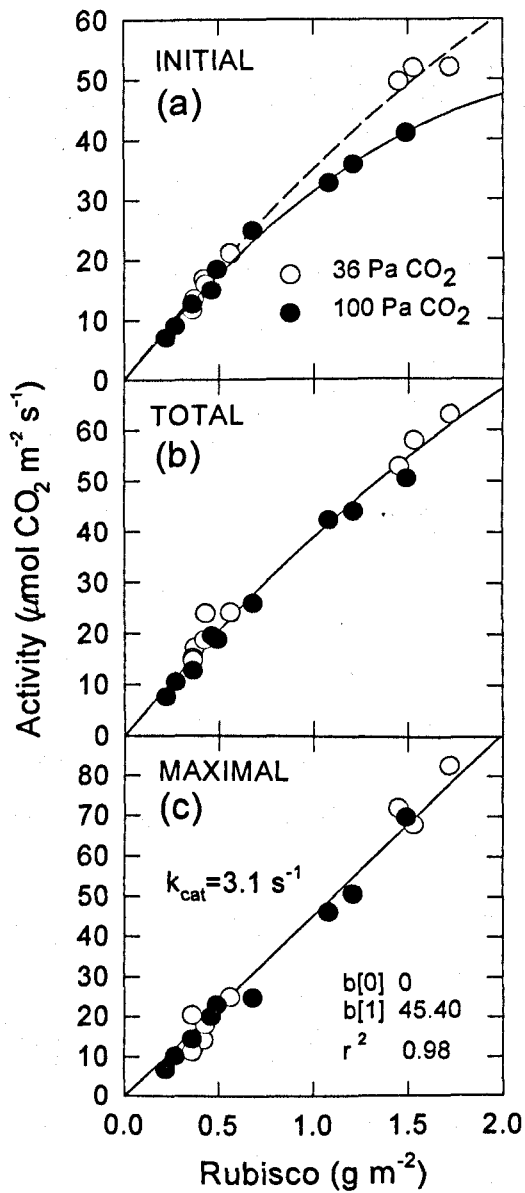


the real situation, since the maximal activity measured in the 36 Pa CO<sub>2</sub> treatment was lower than the total activity, suggesting an analytical error on this occasion.

#### 4.7.3 Results – Activity of Rubisco as a function of total Rubisco content

Figure 4.13 below, shows the relationships between estimates of *in vitro* Rubisco activity versus Rubisco content estimated by the binding of [<sup>14</sup>C]CABP, both of which were measured in the same individual samples. The initial and total activities (Figure 4.13 a & b respectively) show distinct curvilinear relationships with the amount of Rubisco, whilst the maximal activity is proportional giving an estimated  $k_{cat}$  of 3.1 s<sup>-1</sup> at 25°C. Whilst the relationships between total and maximal activity and Rubisco content are independent of growth CO<sub>2</sub> treatment, the lines for initial activity diverge at higher Rubisco contents, such that at a Rubisco content of 1.5 g m<sup>-2</sup>, the initial activity is 16% lower for the 100 Pa CO<sub>2</sub> grown treatment compared to 36 Pa CO<sub>2</sub> grown.

If we consider a Rubisco content of 1.5 g m<sup>-2</sup>, we observe that for the 36 and 100 Pa CO<sub>2</sub> grown plants, initial activities are 71 and 59% of maximal respectively, and that the same total activity for both CO<sub>2</sub> treatments, is 78% of maximal. Clearly, as Rubisco content declines, so these differences between initial, total and maximal activities diminish, so that at less than 0.5 g m<sup>-2</sup> Rubisco, all the enzyme is fully carbamylated, and no inhibitor(s) is/are present.



**Figure 4.13** Estimates of *in vitro* Rubisco activity versus Rubisco content (both measured in the same individual samples), in flag leaves of different ages measured between 8 d before until 18 d after anthesis in Experiment Four. Plants were grown at 36 (open symbols) and 100 (closed symbols) Pa CO<sub>2</sub> partial pressure. Initial, total and maximal activities were determined at 25°C on Rubisco isolated from leaves freeze clamped under a PPFD of 1500 μmol quanta m<sup>-2</sup> s<sup>-1</sup> and the growth  $pC_a$ . Lines are regressions forced through the origin. Separate regressions were used for the two treatments where this improved the R<sup>2</sup> value (dashed lines), otherwise a single regression was used (solid line). Second order linear regressions were used where this improved the R<sup>2</sup> value (Initial and Total), otherwise first order regression was used.

#### 4.7.4 Discussion

It is generally assumed that Rubisco in excess of that required to maintain carboxylation rates will become deactivated, since RuBP concentrations are generally

stable (Sage, 1990). In support of this, decreases in Rubisco activation state to elevated  $\text{CO}_2$  have been found in many studies (Spencer and Bowes, 1986; Vu et al., 1987; Sage et al., 1989; Rowland-Bamford et al., 1991), but not in all (Sicher, 1995; Nakano et al., 1997), and further, similar decreases in Rubisco activation have been observed in plants grown under limiting irradiance (Parry et al., 1997) or in plants subjected to source-sink manipulation (Sawada et al., 1995). The data presented in Figures 4.12 and 4.13 for the *in vitro* activities of Rubisco, sampled under saturating irradiance and at the growth  $pC_a$ , support the finding that growth at elevated  $\text{CO}_2$  does decrease the initial activity, and therefore activation of Rubisco. This is clear for the first occasion in Figure 4.12, and in Figure 4.13 where Rubisco contents were highest (and corresponded to the same samples), for which initial activity was 17% lower in the 100 Pa  $\text{CO}_2$  compared to 36 Pa  $\text{CO}_2$  grown plants. However, unexpected was the decreased activation of 36 Pa  $\text{CO}_2$  grown plants of 60 to 70% at high Rubisco contents, since it has been assumed that all Rubisco is utilised at high light and 36 Pa  $\text{CO}_2$ , for which there is good evidence (Makino, 1988; Sage, 1990). It has previously been demonstrated (section 3.2.2) that the light intensity of  $1500 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$  used in the harvesting of samples in this experiment was saturating for this cultivar at 36 Pa  $\text{CO}_2$ . Part of the decrease in activation state (78 and 56% respectively of the difference for 36 and 100 Pa  $\text{CO}_2$  treatments at  $1.5 \text{ g m}^{-2}$  Rubisco) is associated with an inhibitor(s), demonstrated by the difference between total and maximal activity (Parry et al., 1997). Sceptics of the maximal activity assay might argue that the measurement is merely a reflection of an increase in Rubisco protein, as a proportion of the total present, during desalting (see method, section 4.5.2), and this would of course largely account for the unexpected inactivation of 36 Pa  $\text{CO}_2$  grown plants. However Parry et al., (1997, Table 1), clearly demonstrate that this is not the case,

given that extracts passed through a desalting column and then activated with  $\text{HCO}_3^-$  and  $\text{Mg}^{2+}$  for 3 minutes, did not have significantly different activities from controls activated in the same way but not desalted. In fact, despite the lack of significance, desalted samples had lower activities (approximately 10%), suggesting that even with this method, maximal activities may still be slightly underestimated. That the maximal activity observed may be a significant artefact of Rubisco activity stimulation by  $\text{SO}_4^{2-}$  ions (as reported by Moore and Seeman, 1994), was also discounted (Parry et al., 1997). Thus, it is interesting to note that since 36 and 100 Pa  $\text{CO}_2$  grown plants showed identical responses for total and maximal activities (Fig. 4.13), the amount of Rubisco inactivated by the presence of an inhibitor(s) was independent of  $\text{CO}_2$ .

An additional part of the decrease in activation state for the 36 Pa  $\text{CO}_2$  grown plants, arises from the difference between initial and total activity. Initial activity is known to be affected by the pre-treatment of the extraction buffer and time taken to determine activity (Sage, 1993), which may have lowered absolute measurements of initial activity here. However, if this is the case, then we might expect an equal percentage loss of initial activity in all samples, and therefore a linear response of initial activity to Rubisco content, with lower initial activities than total, at much lower Rubisco contents. This observation however, is not apparent. What is interesting, is that the response of the various activities and the resultant activation for 36 Pa  $\text{CO}_2$  grown plants in this experiment, closely mirror those observed in the preliminary experiment discussed previously (section 4.5.3, Fig. 4.5).

The *in vitro* maximal activity of Rubisco was closely correlated ( $R^2=0.98$ ) with amount, and the estimated  $k_{\text{cat}}$  of  $3.1 \text{ s}^{-1}$  is the same as that estimated previously for wheat at  $25^\circ\text{C}$  (Makino et al., 1988), which gives further support to the reliability of the maximal activity assay.

## 4.8 Conclusions

From results obtained over the course of three separate experiments in which leaf samples were taken from full emergence through to senescence, the long-term growth of *T. aestivum* L. cv Minaret at 70 compared to 36 Pa CO<sub>2</sub>, had no significant effect on the amounts of Rubisco or leaf N prior to at least six days after anthesis. Thereafter, some significant effects between the two CO<sub>2</sub> treatments were observed, with amounts of Rubisco and N being decreased in the 70 Pa CO<sub>2</sub> treatment. However, there was no evidence for a preferential decline in the amount of Rubisco as a proportion of total leaf N content on any occasion, from which we can surmise, based on the components selected for measurement, that there was not an ideal biochemical acclimation to elevated CO<sub>2</sub> in these plants. This was despite a 17% decrease in the initial activity of Rubisco in plants shortly after full leaf expansion and grown and sampled at 100 Pa CO<sub>2</sub> and saturating light, suggesting that the carboxylation capacity, and therefore amount of Rubisco was in excess of requirements.

Further, long-term growth at 70 Pa CO<sub>2</sub> had no significant effect on the relationships of Chl, Chl a/b ratio or soluble protein as a function of leaf N. Given this fact, and the early but parallel loss of Rubisco and N in 70 Pa CO<sub>2</sub> grown leaves, this suggests that leaves grown at elevated CO<sub>2</sub> did senesce slightly earlier.

A similar absence of any effect of elevated CO<sub>2</sub> on the amount of leaf components as a fraction of leaf N at full emergence, has previously been reported for wheat (Makino, 1994), and more recently for rice (Nakano et al., 1997). Further, Nie et al., (1995a) in a FACE study of wheat have reported that protein composition was unaltered between 35 and 55 Pa CO<sub>2</sub> grown flag leaves at full expansion, and Delgado

et al., (1994) reported that amounts of Chl, soluble protein and Rubisco per unit leaf area did not change in winter wheat exposed to elevated CO<sub>2</sub>.

In accordance with observations of Nie et al., (1995a), in the experiments reported here, effects of elevated CO<sub>2</sub> were consistently seen subsequent to anthesis, in which senescence was enhanced. However, here the similarities end, since no evidence for a CO<sub>2</sub> dependent decline in the amount of Rubisco was found in this study, contrary to the observations of Nie et al., (1995a), in which such an effect was reported to have occurred during senescence. Nakano et al., (1997) also reported a CO<sub>2</sub> dependent decline in the amount of Rubisco in rice, but demonstrated that this was entirely due to decreased N content at elevated CO<sub>2</sub>, given that various leaf components decline with different proportionalities to leaf N content, as has been demonstrated here for Chl, soluble protein and Rubisco. Subsequently, in order to investigate these relationships further, and to try and explain the contrasting observations for wheat, it was decided that the response of the thylakoid-ATPase to elevated CO<sub>2</sub> would be studied, and this investigation forms the topic of the next chapter.

## CHAPTER 5

### The long-term effect of elevated partial pressures of CO<sub>2</sub> on the amount of Coupling Factor 1

#### 5.1 Introduction

As discussed in detail in the introductory chapter, a long-term increase in  $pC_a$  will result in an imbalance between the capacities for carboxylation and RuBP-regeneration, which will represent an inefficient use of resources unless an inherent mechanism exists and operates to redress the balance. One way in which this could be achieved, is through a specific decrease in the amount of Rubisco as a proportion of leaf N, but as we have seen in the preceding chapter, there is little evidence for this in the wheat plants grown in this study.

However, it could still be the case that the imbalance between capacities might be overcome, if the amounts of components which determine RuBP-regeneration increase as a proportion of total leaf N (Webber et al., 1994). Evidence suggestive that such a response occurred, was obtained in an experiment in which winter wheat was grown at 35 and 70 Pa CO<sub>2</sub> and at N applications of 87 or 489 kg ha<sup>-1</sup> (Delgado et al., 1994). Leaves grown at 70 Pa CO<sub>2</sub> and surprisingly at the low as well as the high N application, had greater photosynthetic capacities (10% mean increase over the experiment) than those grown at 35 Pa CO<sub>2</sub>, and there were no differences in carboxylation efficiency.

In another experiment with spring wheat grown with an unlimited N supply, Habash et al., (1995) observed 16 and 15% increases in the initial and total activities of Rubisco respectively, a corresponding 15% increase in the *in vivo* carboxylation efficiency, a higher relative quantum yield of CO<sub>2</sub> fixation ( $\Phi_{CO_2}$ ), and significantly

higher assimilation rates at all  $pC_i$  values measured for young plants grown at 70 compared with 35 Pa CO<sub>2</sub>. However, one interpretation of this could be that greater root growth in the elevated CO<sub>2</sub> environment allowed greater N uptake and higher leaf N and Rubisco contents. Further, in both experiments, amounts of components which determine RuBP-regeneration capacity were not measured.

Like the observations of Delgado et al., (1994) and Habash et al., (1995), there was some but by no means conclusive evidence from the gas exchange studies of Chapter Three, to suggest that a partial re-balancing between capacities may have occurred late in ontogeny in leaves of plants grown at elevated  $pC_a$ . Since gas exchange studies only ever reflect the activity of components, the possibility that greater adjustments to the amounts of components as a proportion of leaf N occur, but are masked by changes in the degree of activation cannot be ruled out (i.e. increases in the proportion of leaf N invested into RuBP-regeneration components could be accompanied by decreased percentage activation). Further, the low N treatment used by Delgado et al., (1994) for plants in which a stimulation of photosynthetic capacity was apparently observed, likewise fell within the range of N treatments used in this study.

Thus, the aim of the work reported in this chapter, was to determine whether long-term growth of spring wheat at 70 Pa CO<sub>2</sub> would have any effect on the amount of coupling factor 1 (CF<sub>1</sub>) of the thylakoid ATP-ase, relative to leaf N and Rubisco contents.

Coupling factor 1 was chosen, because in terms of N investment, the ATP-ase of which it forms a substantial part, is the single largest component which determines RuBP-regeneration capacity, typically accounting for 5.4% of total leaf N in C<sub>3</sub> sun leaves (Evans and Seeman, 1989). Additionally, the thylakoid ATP-ase



occupies a pivotal role in chloroplasts by providing ATP not only for photosynthetic carbon reduction, but for the reassimilation of ammonia from photorespiration, and the reduction of nitrite and sulphate amongst others (Wallsgrave et al., 1983). However, it is believed that C reduction provides the vast majority of sink for the consumption of ATP and reducing power (Habash et al., 1995). In other species where relationships between the amount of CF<sub>1</sub> and photosynthesis has been studied, the thylakoid ATP-synthase or CF<sub>1</sub> component have always been expressed on a relative basis (Makino et al., 1997; Nakano et al., 1997). Thus a further aim was to develop a method for accurately quantifying CF<sub>1</sub>, and to learn something of the relationship between this component and other parameters in wheat.

In this chapter, methods and the testing of assays for determining (1), the total activity and (2) the amount of CF<sub>1</sub> are described. Data from two separate experiments relating to the long-term effect of elevated CO<sub>2</sub> on the amount of CF<sub>1</sub> and from the same leaf samples, amounts of Chl and soluble protein are then presented, first on an area basis as a function of leaf age, and then on a leaf area basis as a function of leaf N content. Finally, conclusions will be drawn as to whether or not there was a specific CO<sub>2</sub> effect on the amount of CF<sub>1</sub> in leaves of wheat grown in these experiments.

## **5.2 Determination of Mg<sup>2+</sup>-specific ATP-ase activity**

The method described here for determining the Mg<sup>2+</sup>-specific ATP-ase activity in wheat, is based on that applied to lettuce by Pick and Bassilian (1981), and relies on the ability of the detergent octyl glucopyranoside at concentrations of 30 to 40 mM, to fully activate the coupling factor (CF<sub>1</sub>) subunit of this enzyme. With

activation, CF<sub>1</sub> catalyses the hydrolysis of ATP to ADP and PO<sub>4</sub><sup>3-</sup>, with the amount of the latter released being measured by the method of Lanzetta et al., (1979). In addition to lettuce, the method has previously been applied to pea (Leong and Anderson, 1984; Evans, 1987) and spinach (Evans and Terashima, 1987). The basic method is presented first, with subsequent discussion of modifications made for the determination of ATP-ase activity in wheat.

#### 5.2.1 *Basic method for determining the Mg<sup>2+</sup> specific ATP-ase activity in wheat*

##### *Materials and Method*

At 0 to 4°C, leaf material (approximately 600 mm<sup>2</sup>) was homogenised with a blade homogeniser for 30 s in 2 mL of a 50 mM Bicine buffer (pH 7.6), containing 400 mM Sorbitol, 20 mM MgCl<sub>2</sub>, 1 mM EDTA, 50 mM β-Mercaptoethanol and 5 mM PMSF. Two 50 μL aliquots of the homogenate were removed and stored on ice in the dark, prior to the determination of total chlorophyll content (section 2.4). Chloroplasts were pelleted at 3000 xg for one minute, the supernatant discarded, and then chloroplasts resuspended in 2 mL of the same Bicine buffer described above, but with a decreased concentration of Sorbitol (100 mM). After five minutes on ice to osmotically shock chloroplasts, thylakoids were pelleted at 10000 xg for two minutes, after which the supernatant was removed and thylakoids gently resuspended in 1 mL of a 10 mM Tricine-NaOH buffer (pH 7.6), which contained 100mM Sorbitol, 50 mM KCl and 50 mM NaCl. Two further 50 μL aliquots were removed at this stage, to determine the thylakoid chlorophyll content.

For the enzyme assay, 150  $\mu\text{L}$  of each sample (10-20  $\mu\text{M}$  Chl) was added to 500  $\mu\text{L}$  of a 50 mM Tricine-NaOH (pH 7.6) buffer, which additionally contained 40 mM octyl glucopyranoside, 2 mM  $\text{MgCl}_2$  and 0.1 mM EDTA, and was pre-incubated at 37°C for five minutes. The reaction was started by adding ATP to 4 mM. In early trials of the assay, reactions were stopped after 10 minutes, but following subsequent investigation (see 5.2.3 & 5.2.4 below), incubations were stopped after two minutes. This was done by quenching with 150  $\mu\text{L}$  of 10% TCA. Assays included blanks without thylakoids to determine the phosphate released from the ATP by TCA, and sample blanks in which TCA and then the ATP were added, in order to determine the amount of free background phosphate in extracts. Following the assay, samples and blanks were deproteinised at 3000  $\times g$  for two minutes and the supernatants retained.

To determine the amount of phosphate present, 50  $\mu\text{L}$  of supernatant or distilled water as controls was added to 950  $\mu\text{L}$  of distilled water in a disposable plastic cuvette, to which 200  $\mu\text{L}$  of ammonium molybdate reagent was added, mixed, and left to stand. The ammonium molybdate reagent had previously been prepared by dissolving 8.75 g of ammonium molybdate.4 $\text{H}_2\text{O}$  in 400 mL of distilled  $\text{H}_2\text{O}$ , to which 36 mL of 18 M  $\text{H}_2\text{SO}_4$  was cautiously added, and upon cooling to room temperature, was made up to 500 mL with distilled  $\text{H}_2\text{O}$ .

After standing for 10 minutes, 200  $\mu\text{L}$  of a Malachite green reagent was added to each cuvette, mixed, and left to stand. This reagent had previously been prepared by boiling 600 mL of distilled water, cooling it to 80°C, and dissolving 1.75 g of polyvinyl alcohol in 400 mL. This was cooled to room temperature, upon which

0.175 g of Malachite green was added, and the reagent volume brought to 500 mL, with some of the remaining and previously boiled distilled H<sub>2</sub>O.

After 30 minutes, 200  $\mu$ L of 1 M Citric acid was added to each cuvette and mixed to stop any further reaction. Absorbencies were read at 610 nm, and phosphate concentrations in samples calculated with reference to a standard curve made with K<sub>3</sub>PO<sub>4</sub>.

#### *Preliminary assessment of method*

To test the assay and its applicability to wheat samples, preliminary studies were made, for which plants of *Triticum aestivum* L. cv Minaret were grown six to a 160 mm diameter pot in compost, on a glasshouse bench with a day/night temperature of 16/8°C, and supplemental light to give a 16 h photoperiod. Plants were watered daily and received ample nutrients. The aim of the first study was to assess how activity might vary from samples taken at different positions along the length of the same individual leaf, since this could have important implications for deciding which particular section of a leaf to clamp during gas exchange analysis (see Chapter Three). Subsequent to stem extension, young and recently fully expanded leaves of approximately 300 mm in length were each sampled at two positions; 30 mm from the tip and 30 mm from the base. At these positions, leaf sections of approximately 300 mm<sup>2</sup> were removed and assayed using the method described.

#### *Results and Discussion*

Whilst not significantly different ( $\alpha=0.05$ ;  $P=0.266$ ), the activity decreased on average by 30.2% ( $\pm 10.4$ ), in moving from the younger base of leaves towards older tips (Table 5.1), verifying the need for caution and consistency during gas exchange

analysis for sampling comparable regions for treatment replication. It is likely that the third replicate leaf chosen (Table 5.1), which had much lower absolute values at both leaf positions, contributed to a lack of significant difference in the overall findings.

Replicate Number	CF <sub>1</sub> hydrolytic activity (mmol PO <sub>4</sub> <sup>3-</sup> mol <sup>-1</sup> Chl s <sup>-1</sup> )		% Difference
	30mm from leaf base	30mm from leaf tip	
1	20.6	15.4	30.2
2	21.3	10.6	50.2
3	9.3	7.9	15.1
Mean	17.1 (±3.9) <sup>a</sup>	11.3 (±2.2) <sup>a</sup>	30.2 (±10.4)

**Table 5.1** Comparison of the CF<sub>1</sub> hydrolytic activity in samples taken at the base and tip region of the same individual leaves of spring wheat. The mean of three independent estimations ± Std Err are shown from leaves of comparable length and ontogeny (although see text above); different superscript characters indicate statistical differences between columns at  $P \leq 0.05$ .

However, regardless of specific sampling position, of greater concern were the very low absolute values obtained by using this method. In Evans and Terashima (1987) for example, values ranged from 528 to 627 mmol PO<sub>4</sub><sup>3-</sup> mol<sup>-1</sup> Chl s<sup>-1</sup> for samples of young fully expanded spinach leaves grown at a range of nitrate concentrations, but otherwise optimal conditions. In young pea leaves grown at a range of irradiances but otherwise optimal conditions (Evans, 1987), activity values obtained ranged from 160 to 500 mmol PO<sub>4</sub><sup>3-</sup> mol<sup>-1</sup> Chl s<sup>-1</sup>. Since wheat leaves generally have higher photosynthetic capacities than those of spinach or pea grown under optimal conditions and measured at comparable ontogeny (Evans, 1989), one might expect similar if not greater absolute values to be obtained for CF<sub>1</sub> hydrolytic activity in this species. This suggested that the activity in wheat measured here, was being considerably underestimated.

In an attempt to understand where possible losses of activity might have been occurring, further investigations were made, and these are presented and discussed below. In all instances and unless stated otherwise, material used was obtained from the wheat plants grown in the glasshouse as described above, and the assay was done according to the protocol at the beginning of this section, with homogenisation and extraction steps at 0 to 4°C.

#### 5.2.2 *The effect on CF<sub>1</sub> hydrolytic activity, of standing extracted samples on ice for prolonged periods prior to assay*

##### *Introduction*

This question was important, not only from consideration of whether proteolytic degradation might be occurring, as discussed previously in section 4.2.2 for Rubisco samples, but also through a desire, given the number of potential samples to analyse if the assay proved reliable, that would need to be extracted and assayed in batches. Thus some understanding of working constraints were necessary.

##### *Materials and Method*

Three replicate leaf samples of similar length and ontogeny were individually homogenised and the brei from each divided in two. One half of the brei was assayed immediately (control), and the second assayed after a period of 60 minutes standing on ice (treatment).

## Results

With the exception of the first replicate leaf (Table 5.2 below) which showed a 14% decrease in activity after 60 minutes of standing on ice, when all data were combined there was no significant effect of this treatment on samples ( $\alpha=0.05$ ;  $P=0.871$ ).

Leaf replicate	mmol PO <sub>4</sub> <sup>3-</sup> mol <sup>-1</sup> Chl s <sup>-1</sup>	
	Control	Treatment (60 min on ice)
1	72.0	62.0
2	25.0	25.0
3	28.0	28.0

**Table 5.2** The effect on CF<sub>1</sub> hydrolytic activity of standing homogenised wheat leaf samples on ice for 60 minutes prior to assay, compared to controls from the same individual leaves which had been assayed immediately.

## Discussion

This finding was consistent with results for Rubisco samples treated in the same way (section 4.2.2), and suggests that degradation through proteolysis was not a major problem while extracted samples were stored on ice. However, it should be noted that it was never necessary to leave extracted CF<sub>1</sub> samples on ice for periods of greater than 30 minutes during the course of these preliminary studies. Nevertheless, despite no apparent loss of activity in homogenates, absolute values still remained low, so the next step was to test aspects of the activity assay.

### 5.2.3 *The effect of assay incubation temperature and duration on CF<sub>1</sub> hydrolytic activity.*

## Introduction

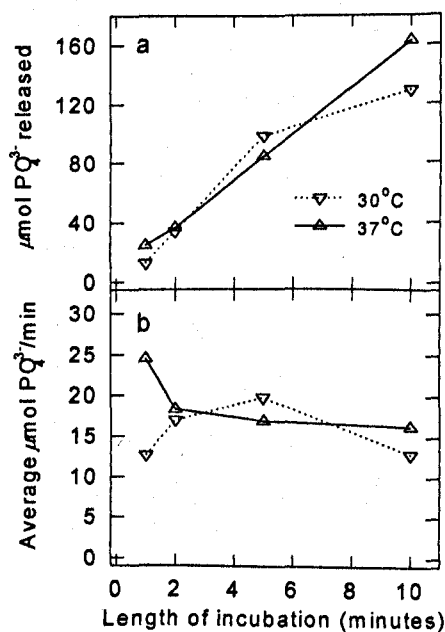
It was considered that the low absolute values for activity could possibly be caused by the comparatively high incubation temperature (37°C) employed, which it

was postulated, might lead to an increased activity of proteases which may have been present in the reaction mixture, but not previously active at lower temperatures (i.e. when samples were on ice).

### *Materials and Method*

In a cold room, several leaf samples from the glasshouse grown plants were finely chopped and combined to form a single homogeneous sample, which was promptly homogenised and divided into ten equal aliquots. Half of these were assayed at 30°C and half at 37°C. At each temperature, one aliquot was assayed for zero time as a control, a second for one minute, and further aliquots for two, five and ten minutes.

### *Results and Discussion*



**Figure 5.1** The response of wheat CF<sub>1</sub> hydrolytic activity (μmol PO<sub>4</sub><sup>3-</sup> released) to increased length of incubation at two temperatures as denoted by the key (a), and (b) the same activity but plotted as the rate per minute against the length of incubation at both temperatures.



Figure 5.1 (a) shows the response of the concentration of  $\text{PO}_4^{3-}$  released with increasing sample incubation time, for which there is little evidence for an effect of incubation temperature, demonstrating that the higher temperature was not deleterious to enzyme activity. The amount of  $\text{PO}_4^{3-}$  released per minute was fairly constant for different incubation times, but for a slight decline with increased incubation likely due to random error (Fig. 5.1 b).

At both temperatures, a rapid rise in the release of  $\text{PO}_4^{3-}$  was observed, reaching a maximum after one minute at  $37^\circ\text{C}$  and five minutes at  $30^\circ\text{C}$  (Fig 5.1 b), declining thereafter at both temperatures. At the time it was considered that this loss of activity could have been due to the presence, either of metabolites or other chemicals which interfered with the active sites of  $\text{CF}_1$ , or alternatively by proteases activated by the high incubation temperature. However, it was considered that the non-linearity was unlikely to be caused by substrate limitation, since while the concentration of ATP in the stroma is typically 1.5mM (Lawlor, 1993) and in the assay mixture only slightly greater than this (4mM ATP) was provided, the thylakoids will have been diluted at minimum 1000-fold in the extraction process, so that ATP should still have been vastly in excess *in vitro*. Thus from this brief study, it was concluded that incubation at  $37^\circ\text{C}$  was an appropriate temperature to use for this assay and species, but that possibly, the reaction was tailing off with time. The latter is considered further in the next experiment.

#### 5.2.4 *The effect of spiking assayed samples*

##### *Introduction*

In order to test the reliability of the final assay for determining the amount of  $\text{PO}_4^{3-}$  released by samples incubated in the presence of octyl glucopyranoside and

ATP, samples were assayed either with or without an additional 15  $\mu\text{M}$  spike of  $\text{PO}_4^{3-}$ .

### *Materials and Method*

A collection of leaves were ground as one homogenous sample and divided into six equal aliquots, and then incubated for zero, one, two, five or ten minutes at 37°C. Subsequent to quenching each sample was divided in two, to one of which a spike of 15  $\mu\text{mol}$   $\text{PO}_4^{3-}$  was added prior to determination of the  $\text{PO}_4^{3-}$  concentration in all samples, by the method described in section 5.2.1 above. Blanks without thylakoids were included for each incubation to determine the phosphate released non-enzymically from ATP by TCA. Additionally, two aliquots were used for zero time. For one, TCA was added before the ATP (0+), and in the other, the ATP was added immediately prior to TCA (0).

### *Results and Discussion*

Incubation time (mins)	$\mu\text{mol PO}_4^{3-}$				
	Sample blank	Sample	Sample minus blank	"Spike" minus blank	"Spike" recovered
0+	91.7	98.1	6.4	17.6	11.3
0	93.7	104.2	10.5	20.8	10.3
1	84.6	114.9	30.3	43.2	12.9
2	83.3	129.9	46.6	59.0	12.4
5	82.7	185.5	102.8	127.8	25.0
10	60.3	263.5	203.2	231.3	28.1

**Table 5.3** The response of the release of  $\text{PO}_4^{3-}$  from ATP hydrolysed by isolated wheat  $\text{CF}_1$ , to increased incubation time (columns 1-4 (L-R)), and recovery of  $\text{PO}_4^{3-}$  added to 15  $\mu\text{M}$  as a spike to an aliquot of the same samples (columns 5&6). 0+ =zero time incubation in which TCA was added prior to ATP, and conversely 0 =zero time sample in which ATP was added immediately prior to TCA. All samples were incubated at 37°C. Sample blanks contained no thylakoids, but otherwise, all assay reagents.

Curiously, the concentration of  $\text{PO}_4^{3-}$  released from ATP upon the addition of TCA to blanks without thylakoids, decreased with longer incubations (Table 5.3, second column), presumably due to a slow non-enzymatic hydrolysis of ATP releasing  $\text{PO}_4^{3-}$  during the incubation period.

The difference in concentration of  $\text{PO}_4^{3-}$  released from ATP upon addition of TCA in the sample blanks, and that of samples (column four, Table 5.3 above) will represent the amount of ATP hydrolysed and  $\text{PO}_4^{3-}$  released by  $\text{CF}_1$  during the assay, plus an additional component representing the free background  $\text{PO}_4^{3-}$  concentration in the original extract. The latter can be deduced by the difference between the sample incubated at zero plus (0+) time in which TCA and then ATP was added, and the blank for this sample, which in this instance, results in a value of  $6.4 \mu\text{M}$   $\text{PO}_4^{3-}$  as the free concentration (Table 5.3, column four). Presumably, since all subsamples originated from the same homogeneous leaf sample in this experiment, all have the same free background  $\text{PO}_4^{3-}$  concentration. It follows, that the difference in concentration of  $4.1 \mu\text{M}$   $\text{PO}_4^{3-}$ , between incubation time 0+ and zero (0), in which the ATP and then TCA was added a fraction of a second later (Table 5.3, column four), represents  $\text{PO}_4^{3-}$  released enzymatically during this very short period. Therefore, for obtaining an accurate sample zero, and also an accurate estimate of free  $\text{PO}_4^{3-}$  in the cellular extract, TCA was always added before ATP in subsequent assays, since the order of addition clearly affects the final estimation.

On average, the spike of  $15 \mu\text{M}$   $\text{PO}_4^{3-}$  added to each sample was fully recovered as  $16.7 (\pm 3.2 \text{ Std Err}) \mu\text{M}$   $\text{PO}_4^{3-}$ , but for individual samples represented recoveries of 69 to 187% (Table 5.3, column six). If one allows for pipetting errors given the small volumes involved, and the reproducibility on different days of

standard curves for the assay (not shown), this suggests that the  $\text{PO}_4^{3-}$  assay was sufficiently reliable.

At this stage, further investigation had demonstrated that extracted samples were stable on ice for at least 60 minutes, incubation at  $37^\circ\text{C}$  enhanced hydrolytic activity, and the  $\text{PO}_4^{3-}$  assay was reliable. There was however a problem with activity tailing off with longer incubations, and so as a compromise between this and the possibility that longer incubations appeared to underestimate sample blanks (Table 5.3, column two), a standard assay incubation time of two minutes was decided upon, and has been applied in all subsequent assays.

#### 5.2.5 *Pea versus Wheat: a comparison of $\text{CF}_1$ hydrolytic activity*

##### *Introduction*

Despite some improvements to the basic assay method, the fundamental problem still persisted that absolute activity values for wheat  $\text{CF}_1$  were considerably lower than those required to support observed photosynthetic rates, and were lower than values obtained previously in pea or spinach. Thus it was decided to apply the assay to material of both wheat and pea, to try and determine whether problems were more intrinsic of the assay or the material being used.

##### *Materials and Method*

Seeds of *Pisum sativum* L. cv Kelvedon Wonder were pre-germinated on damp tissue paper in a petri-dish for 48 hours at  $25^\circ\text{C}$  prior to sowing in pots of compost. These plants were then grown alongside wheat plants in a glasshouse, for which growth conditions have previously been described. At 21 d, samples of the top three

unfolded leaflets of pea (selected exactly as described by Evans (1987)), and recently fully expanded sixth mainstem leaves of wheat were chosen, and analysed using the CF<sub>1</sub> assay with the modification of a two minute incubation as discussed at the end of the last section. The wheat leaves were of comparable length (approx. 250 mm) and ontogeny. From each, in the same relative position, 600 mm<sup>2</sup> of leaf was removed for analysis, to gain some idea of the variation of activity between leaves of comparable ontogeny.

### *Results and Discussion*

Coupling factor hydrolytic activity was almost three-fold higher in young pea leaves compared to young wheat leaves (Table 5.4, below), with absolute activities for pea CF<sub>1</sub> being close to the bottom range of those obtained in pea by Evans (1987; 160 to 500 mmol PO<sub>4</sub><sup>3-</sup> mol<sup>-1</sup> Chl s<sup>-1</sup>). This suggested that the major problem with the assay and its applicability to wheat samples, most likely resided with the initial extraction procedure and characteristic problems of extracting protein from the leaves of this particular species. While the assay appeared to underestimate absolute values in wheat, it showed good consistency in the small standard errors for both wheat and pea replicates (Table 5.4).

Replicate	CF <sub>1</sub> hydrolytic activity (mmol PO <sub>4</sub> <sup>3-</sup> mol <sup>-1</sup> Chl s <sup>-1</sup> )	
	<u>Wheat</u>	<u>Pea</u>
1	53.4	128.9
2	50.1	121.3
3	37.0	138.4
4	40.1	-
Mean	45.2 (±3.9)	129.5 (±4.9)

**Table 5.4** Comparison of CF<sub>1</sub> hydrolytic activity measured in young leaf samples of wheat and pea. The value in brackets is the Std Err.

### 5.2.6 *Sample extraction procedure: A comparison between the current method applied to wheat, and that used by Evans (1987) for pea*

#### *Introduction*

Given that Evans (1987) had obtained much higher values for CF<sub>1</sub> activity in pea, a comparison was made between his extraction protocol and that currently reported, to determine if activities recovered from wheat could be further improved.

#### *Materials and Method*

In a cold room, one 60 mm long section of a recently fully expanded and therefore young wheat leaf was cut into strips approximately 1 mm wide, and these were mixed and split into two equal samples on the basis of FW. One sample was homogenised for 30 s with a blade homogeniser, and then processed in accordance with the standard assay protocol; that is centrifugation of chloroplasts for 1 minute at 3000 xg followed by sedimentation of thylakoids for 2 minutes at 10000 xg. The second sample was processed in essentially the same way, but for adopting the initial extraction method of Evans (1987); a shorter homogenisation of 15 seconds, and longer centrifugation of chloroplasts and thylakoids, both for 5 minutes at 1000 x g. For further comparison, the above was repeated with a section of older wheat leaf material.

#### *Results and Discussion*

Coupling factor hydrolytic activity was always greater in leaf samples extracted using the existing protocol as compared to that of Evans (1987), and in young wheat leaves the difference was significant ( $P < 0.05$ , Table 5.6 below).

Activities in the older leaf samples were always lower than those in the young, regardless of the protocol used. This is not surprising, given the normal decline of components from leaves with increasing ontogeny. It is likely that the shorter homogenisation time used by Evans (1987) was insufficiently long here, to overcome the tougher cell wall nature of even young wheat leaves.

Differences in protocol	Current method	Method of Evans (1987)
Length of homogenisation (seconds)	30	15
Centrifugation of chloroplasts	3000 xg / 1 min	1000 xg / 5 min
Centrifugation of thylakoids	10 000 xg / 2 min	1000 xg / 5 min
Results	CF <sub>1</sub> activity (mmol PO <sub>4</sub> <sup>3-</sup> mol <sup>-1</sup> Chl s <sup>-1</sup> )	
Young leaves	106.8 (±11.1) <sup>a</sup>	47.7 (±1.1) <sup>b</sup>
Old leaves	42.4 (±8.0) <sup>a</sup>	35.7 (±9.3) <sup>a</sup>

**Table 5.6** Comparison between the standard CF<sub>1</sub> homogenisation and extraction protocol, and that previously used by Evans, (1987) for pea, on subsamples from young or old wheat leaves. See text for further details. Values are the mean of two independent assay estimations ± Std Err.

### 5.2.7 *The effect on CF<sub>1</sub> hydrolytic activity of longer homogenisation, filtration, and shortening of the thylakoid centrifugation step*

#### *Introduction*

Based on the conclusion of the last brief study, that the existing method for extracting CF<sub>1</sub> hydrolytic activity worked better for wheat than that used by Evans (1987) for pea, further modifications to the current method of an increased extraction time, presence of a brei filtration step and a shorter centrifugation of thylakoids was investigated.

## *Materials and Method*

In a cold room, one 1200 mm<sup>2</sup> section of recently fully expanded wheat leaf was selected, cut into 1 mm wide strips, these were mixed thoroughly, and then divided into two equal halves on the basis of FW. Using the extraction buffer described in the basic assay method (5.2.1), one subsample of the leaf was homogenised at 0 to 4°C for exactly 30s using a blade homogeniser. After removing aliquots for the determination of Chl content, the homogenate was divided in two, with one of these halves being filtered through 2 layers of Miracloth. Both were then separately centrifuged at 3000 x g for 1 minute, supernatants discarded, and then chloroplasts resuspended in resuspension buffer in accord with the basic method. Following five minutes on ice to lyse chloroplasts, both the filtered and non-filtered samples were each individually halved, one half of each being centrifuged at 10000 xg for two minutes, and the other half of each, at 10000 xg for five minutes. The remaining half of the original leaf sample was processed in exactly the same way, but for an homogenisation lasting exactly 60 s, so that once all the sample preparations were complete, a total of eight sub-samples from the original leaf were obtained, which were then assayed for CF<sub>1</sub> hydrolytic activity using the standard method. The individual procedures for each sub-sample are summarised in Table 5.7 below.

## *Results and Discussion*

From Table 5.7, it is clear that the greatest recovery of activity of 427 mmol PO<sub>4</sub><sup>3-</sup> mol<sup>-1</sup> Chl s<sup>-1</sup> was recorded for sub-sample two, which was homogenised for 30 s, filtered, and then centrifuged for five minutes to recover thylakoids. This magnitude of activity was at the upper range of those previously reported for pea (Evans, 1987), and close to the range reported for spinach (Evans and Terashima,



1987), and supported the previous supposition that activity of wheat CF<sub>1</sub> should be a lot higher than earlier results were suggesting (5.2.1).

Sub-sample	1	2	3	4	5	6	7	8
Homogenisation time (seconds)	30	30	30	30	60	60	60	60
Filtered (Y/N)	Y	Y	N	N	Y	Y	N	N
Thylakoid centrifugation (minutes)	2	5	2	5	2	5	2	5
CF <sub>1</sub> activity (mmol PO <sub>4</sub> <sup>3-</sup> mol <sup>-1</sup> Chl s <sup>-1</sup> )	85.2	427.3	69.7	101.7	213.9	366.9	74.4	88.0

**Table 5.7** Effect of the length of homogenisation, presence or absence of a filtration step, and length of thylakoid centrifugation, on the amount of CF<sub>1</sub> hydrolytic activity recovered in sub-samples of the same original leaf sample.

Considering the results of all subsamples together, with the exception of sub-sample one, the overriding factor which improved recovery, was the inclusion of the filtration step, which presumably removed material of a fibrous nature, so reducing the number of chloroplasts which became disrupted during the first centrifugation, and further, reduced the frequency with which CF<sub>1</sub> may have been knocked off membranes already partially exposed. The second most important improvement came with the longer centrifugation of the thylakoid membranes. The two homogenisation times did not differ consistently.

#### 5.2.8 *Experiment to confirm the reproducibility of the improved activity assay*

##### *Method*

As for the previous experiment (5.2.7), a similarly young fully expanded leaf section of 240 mm<sup>2</sup> was cut into 1 mm wide strips, mixed thoroughly, and then divided into four equal sub-samples on the basis of FW. Each sub-sample was

processed in exactly the same way as for sub-sample two in the previous experiment, with a 30 s homogenisation, a filtration of the brei, and a five minute centrifugation of thylakoid membranes at 10000 xg, the aim of this experiment being to confirm the reproducibility of this approach and establish the level of reproducibility for individual samples.

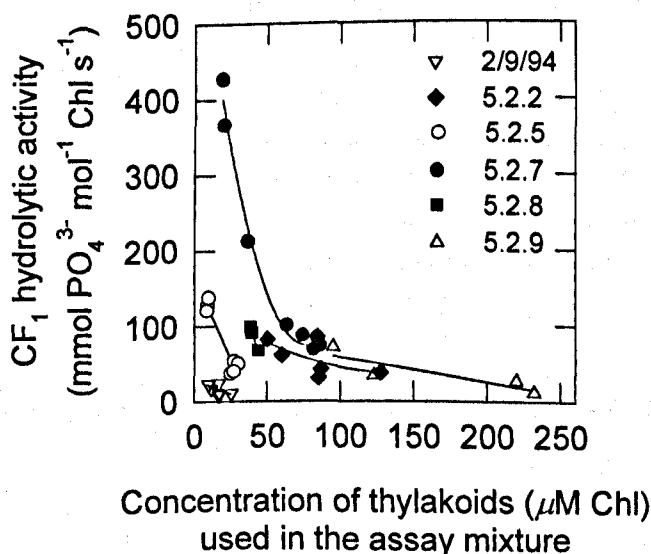
### *Results and Discussion*

The mean CF<sub>1</sub> hydrolytic activity, determined from four separate extractions for this one sample, was estimated at 87.7 ( $\pm 6.6$  Std Err) mmol PO<sub>4</sub><sup>3-</sup> mol<sup>-1</sup> Chl s<sup>-1</sup>, showing satisfactory reproducibility within samples. However, given that the leaf material was grown under the same conditions and was the same age as that used in the previous experiment, the absolute value suggested that there was a major problem with the reliability of this method with use from day to day, since the value obtained was four-fold lower.

#### *5.2.9 Discussion*

At the time of completion of these experiments, it had successfully been demonstrated that the final assay for determining the PO<sub>4</sub><sup>3-</sup> released during incubation was reliable, 37°C was a suitable incubation temperature, and that as for extracted Rubisco samples (4.2.2), the ATP-ase was sufficiently stable in crude homogenates on ice. However, unresolved were the problems of activity tailing off with time (5.2.3), that the activity from blanks not containing thylakoids appeared to decrease with longer incubations (5.2.4), and that collectively, there was considerable day to day variation in absolute values obtained using the assay.

An explanation for some, but not all of these problems has come to light. In the original paper of Pick and Bassilian (1981), in which purified CF<sub>1</sub> at a concentration of 1 mg/mL was used, the authors demonstrated a requirement for a high concentration of octyl glucopyranoside in the assay medium to obtain maximum Mg<sup>2+</sup>-specific activity of CF<sub>1</sub>. They chose to use 40mM octyl glucopyranoside (the concentration also used by Evans and Terashima (1987) and Evans (1987)), in assay media containing thylakoids at 11  $\mu$ M Chl. The same concentration of octyl glucopyranoside was adopted for use in assays here, and in early extractions the concentration of thylakoids recovered were found to be in the range of 9.3 to 16  $\mu$ M, such that no further modifications were considered necessary. However, the subsequent modifications to improve the recovery of activity, increased the concentration of thylakoids (Fig. 5.2, below), and unfortunately, no adjustment was made in the octyl glucopyranoside concentration.



**Figure 5.2** The relationship between CF<sub>1</sub> hydrolytic activity and the concentration of thylakoids in the assay mixture. Data points shown are from some of the experiments discussed in the text above, in sections as denoted by the key. The exception is for data points from a very preliminary experiment done on 2/9/94 which has not been discussed in the text. Lines are plotted for data points within individual experiments, and are only shown to aid interpretation.

Thus, from the data points of the experiments discussed in sections 5.2, 2,7,8 and 9 and shown in Figure 5.2, it is clear that as the concentration of thylakoids in the assay mixture increased, so the activity decreased, presumably because, the concentration of octyl glucopyranoside will have been insufficient to fully activate all the CF<sub>1</sub> present. Therefore, some of the other conclusions in previous sections need to be modified. In section 5.2.5 the apparently three-fold higher activity from pea compared to wheat, was probably largely due to the fact that the concentration of pea thylakoids used in the assay mixture was on average 9.25  $\mu\text{M}$  Chl ( $\pm 0.35$  Std Err) and those of wheat on average three-fold higher at 27.13  $\mu\text{M}$  Chl ( $\pm 1.07$  Std Err). However, this error only accounts for part of the variability in the assay, since from figure 5.2, it is also clear that at a thylakoid concentration of for example 20  $\mu\text{M}$  Chl, activity still varied considerably.

For these reasons, an alternative method for determining CF<sub>1</sub> was sought. This decision was also supported by the knowledge that other workers had encountered problems with the assay, due to variability in the background level of PO<sub>4</sub><sup>3-</sup> released between assays (Dr J.R. Evans, personal communication).

### 5.3 Quantification of Coupling Factor 1

As an alternative to quantifying CF<sub>1</sub> by means of measuring total activity, it was decided to apply the following method in which the amount of this protein in samples would be quantified by comparison against purified wheat standard CF<sub>1</sub>, after electrophoretic separation, transfer to a supporting membrane, and detection by antibodies specific to subunits of the thylakoid ATP-ase. This method was chosen because at the time a similar method for quantifying the delta subunit of CF<sub>1</sub>

of the thylakoid ATP-ase in tobacco had recently been published (Price et al., 1995). Further, this overall approach is much more sensitive than PAGE and densitometric scanning alone, because the process of transferring proteins from the 3D gel matrix onto a 2D supporting membrane allows all the protein of interest to be readily probed by highly specific antibodies (Dunn, 1996; Dr J.R. Evans, personal communication).

In the following pages of this section, a method is first described for isolating and purifying  $CF_1$  from leaves of wheat for use as a standard, followed by a description of the methodology used to separate leaf sample proteins and quantify them, and a description of experiments aimed to test the reliability of the method. Implicit in the switch from measuring activity to amounts of protein, is that nearly all the measured protein can contribute to *in vivo* activity; in this case RuBP-regeneration. This assumption seems reasonable for efficient use of N resources (discussed in Chapter One), and can be justified to some extent by relating amounts of components to gas exchange parameters (see Chapter Six).

### 5.3.1 *Isolation and purification of the ATP-ase coupling factor ( $CF_1$ ) from wheat, for use as a standard*

#### *Materials and Method*

The initial isolation of standard to a purity of approximately 90%, was based on the method described in Moase and Green (1981). Seeds of *Triticum aestivum* L. cv Minaret were sown in trays of compost and grown in a glasshouse for 12 d at a day/night temperature of 16/8°C, and with supplemental light to give a 16 h photoperiod. All steps up to the removal of  $CF_1$  from membranes were done at 4°C,

either in a cold-room or on ice, with all glassware and solutions chilled accordingly. Thereafter, in accordance with Moase and Green (1981), steps were done at room temperature (20 to 25°C), as membrane detached CF<sub>1</sub> is cold labile, although in this particular instance, the recovery of CF<sub>1</sub> which retained activity was not a major isolation criteria.

Leaf blades from approximately 500 plants (roughly equivalent to a total leaf area of 1 m<sup>2</sup>) were removed, quickly weighed, washed in tap and then distilled water, gently blotted dry, and cut into shorter lengths with scissors. Using a Wareing blender, material was homogenised for three bursts of 10 s each, in 5 mL/g FW of isolation buffer which contained 50 mM Tricine (pH 8.0), 0.3 M Mannitol, 1 mM EDTA and 1 mM  $\beta$ -Mercaptoethanol. The brei was filtered through four layers of Miracloth, and chloroplasts were pelleted at 6000 xg for 30 s. The supernatant was discarded, and the pellet resuspended in 1.5 volumes of isolation buffer and pelleted for a second time as before. Chloroplasts were lysed and stromal Rubisco removed, by resuspension and washing of the pellet for 30 minutes in a buffer which contained 10 mM sodium pyrophosphate (pH 7.4), and to which was added protease inhibitors; 6 mM PABA, 40 mM  $\epsilon$ -amino-n-caproic acid and 0.1 mM PMSF. This step was repeated a further two times, with thylakoids pelleted at 10000 xg for 10 minutes between each. During the final wash, the chlorophyll concentration was determined (section 2.4).

At room temperature, the chloroform extraction of CF<sub>1</sub> from thylakoid membranes was performed by a modification of the method of Younis et al., (1977). Thylakoids were suspended between 2.0 to 2.5 mg Chl/mL in 0.25 M Sucrose, 10 mM Tris/SO<sub>4</sub> (pH 7.6), 1 mM EDTA, 2 mM ATP, 5 mM DTT, 6 mM *p*-amino benzoic acid, 40 mM  $\epsilon$ -amino-n-caproic acid and 0.1 mM PMSF. To this was added

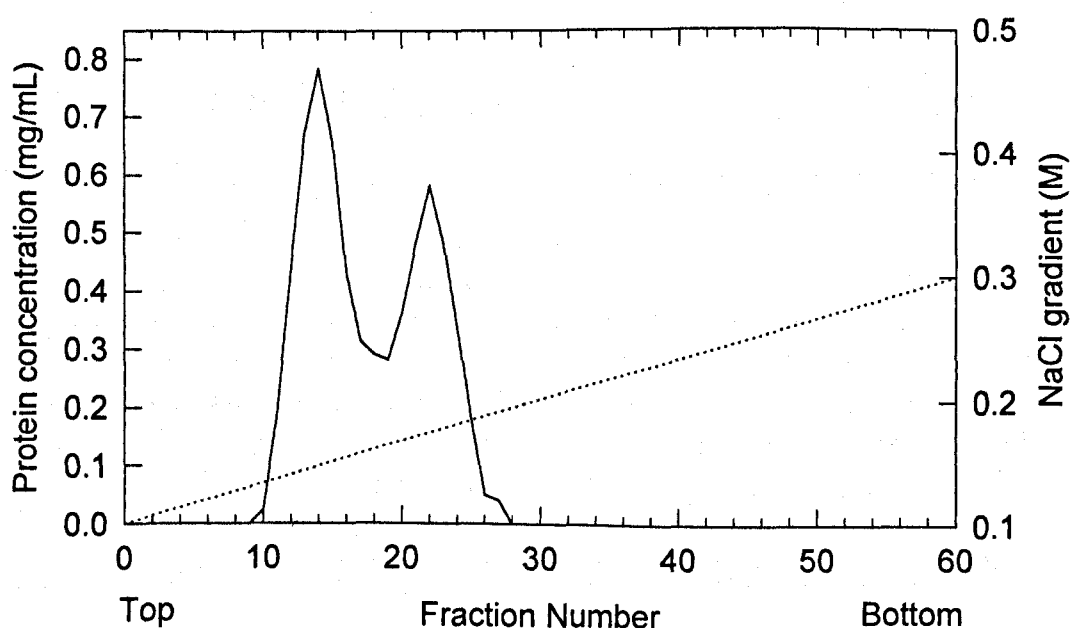
half a volume of chloroform, and the emulsion stirred for 15 s with a magnetic follower. The emulsion was broken by centrifugation at 2500 x g for 1 minute, and the upper aqueous phase (containing the CF<sub>1</sub>) was centrifuged at 48000 xg for 30 minutes. The volume and concentration of soluble protein (section 2.5) in the supernatant was determined, and protein therein was precipitated overnight at 1-2°C by the addition of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, on the basis that 29.5g per 100 mL gives 50% saturation at this temperature.

The following day at room temperature, the crude CF<sub>1</sub> preparation was centrifuged at 6000 xg for 30 minutes, the supernatant removed and centrifuged a second time, from which pellets were resuspended and recombined in a total of 20 mL of buffer 'A' containing 40 mM Tricine NaOH (pH 8.0), 1 mM ATP and 1 mM EDTA.

This crude CF<sub>1</sub> preparation was purified from other contaminating proteins on the basis of size, by gel permeation chromatography (GPC)/molecular sieve chromatography (Preneta, 1989).

Pre-swollen (as supplied by the manufacturer) Sephacryl S-300 (a stable dextran/bisacrylamide matrix) was diluted in eluent (buffer A), which contained 40 mM Tricine-NaOH (pH 8.0), 1 mM ATP and 1 mM EDTA. The resultant slurry was poured into a 300mm glass column of 10mm diameter, and allowed to settle, but never dry out. The solid phase was retained in the column by a frit at the base. To remove any contaminants and settle the column further, it was flushed through with a 100 mL of buffer A, and a second frit was carefully lowered in to form an even surface onto which the protein sample was carefully loaded with a Pasteur pipette. This was washed onto the column with 40 mL of buffer A which

additionally contained 0.1 M NaCl, and which was collected as the wash-on fraction, in a collector (LKB 2112 Redirac) connected to the column via a peristaltic pump with a flow rate of 0.55 mL/minute. Using two 200 mL measuring cylinders, appropriate narrow bore tubing to connect them in series to the column, and a magnetic stirrer in the measuring cylinder closest to the column (cylinder one - mixing chamber), CF<sub>1</sub> in the column was eluted by applying a 0.1 to 0.5 M NaCl gradient. At the start, measuring cylinders one and two each contained 100 mL of buffer 'A' which additionally and respectively contained 0.1 and 0.5 M NaCl. The NaCl gradient, typically used in ion-exchange chromatography, was employed here as a guarantee that all CF<sub>1</sub> would be eluted, given that some charged interactions between matrix and biological molecules have been observed to occur (Preneta, 1989) in GPC.

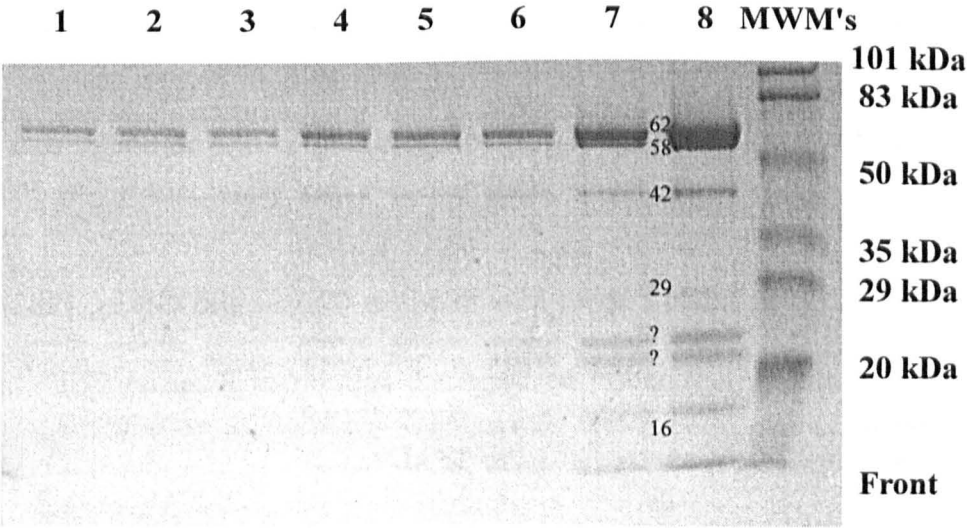


**Figure 5.3** Profile of protein concentration in fractions eluted from a Sephacryl S-300 column, to which a 0.1 to 0.5 M NaCl gradient had been applied in the purification of 'crude CF<sub>1</sub>' extracted from wheat thylakoids.

Fractions were collected at three minute intervals, and their soluble protein contents determined as described in section 2.5, the results of which are shown in



Figure 5.3. Fraction numbers 10 to 16 were pooled as sample one, fractions 17, 18, 19 and 20 respectively became samples two to five, and fractions 21 to 25 were pooled as sample six. These samples plus a sample of the 'crude CF<sub>1</sub>' were solubilised on a SDS:protein basis of 4:1, in a 250 mM Tris buffer (pH 7.6) containing 250 mM Dithiothreitol, 10% (w/v) SDS, 0.2% (w/v) bromophenol blue and 10% (w/v) glycerol, heated for 5 minutes at 90°C, and electrophoresed (10 µg protein per well) on a 12% discontinuous gradient of SDS-polyacrylamide (Bio-Rad Minigel) for one hour at 100 V (variable current). Protein bands were stained in a solution containing 0.1% (w/v) Coomassie Brilliant Blue-R250, 30% (v/v) methanol and 10% (v/v) glacial acetic acid, for one hour on an orbital shaker, after which, the gel was destained overnight in a solution containing 25% (v/v) methanol, 35% (v/v) glacial acetic acid, and 15% (v/v) glycerol.



**Figure 5.4.** SDS polyacrylamide gel electrophoresis of wheat CF<sub>1</sub>, from fractions 10 to 16 in Fig. 5.3, pooled and run as one sample. Variable loadings of this sample are shown; lanes one to three = 1µg, lanes four to six = 2 µg, lane seven 5 µg, and lane eight 10 µg. Lane nine contains molecular weight markers of mass as shown.

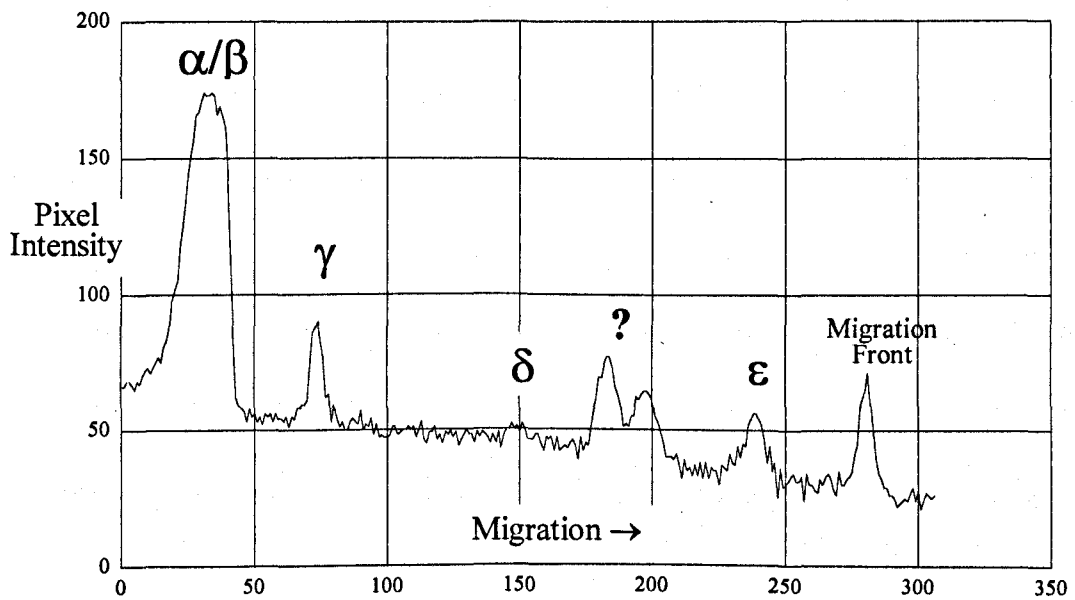
From this gel (image not shown), the purest CF<sub>1</sub> was determined to be from the first sample (fractions 10 to 16). Variable loadings of this fraction, 2 to 10  $\mu$ g, were applied to a second gel (shown above in Figure 5.4) and electrophoresed as just described.

From lane seven in Figure 5.4, wheat CF<sub>1</sub> subunit molecular weights were estimated to be 62 ( $\alpha$ ), 58 ( $\beta$ ), 42 ( $\gamma$ ), 29 ( $\delta$ ) and approximately 16 ( $\epsilon$ ) kilodaltons (kDa). These estimations were consistently 3 to 5 kDa higher for each subunit, when compared to estimations for wheat CF<sub>1</sub> by Moase and Green (1981; 57, 55, 39, 25 and 13 kDa for subunits  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$  and  $\epsilon$ , respectively). These differences most likely reflect variations in the gels used, since on a relative mass basis, both sets of estimations are extremely comparable (see also Figure 2 in Moase and Green (1981)).

The two unidentified bands with molecular weights of 24 and 21 kDa could represent breakdown products of the  $\alpha$  and  $\beta$  subunits. Alternatively, the identification of the  $\delta$  subunit may be incorrect, and may in fact be one of these two unidentified bands, based on the observation that the molecular mass of this subunit can vary from 19 kDa in spinach to 25 kDa in wheat (Moase and Green, 1981). It is unlikely that they are contamination by the small subunit of Rubisco (SSU), as the SSU has a mass range across species of 12 to 18 kDa.

Fortunately, these uncertainties did not affect the calculation of the purity of the sample with respect to the  $\alpha$  and  $\beta$  subunits. This was achieved by first recording the gel image as a TIF file (Sratagene Eagleeye II), and analysing the resultant image with the integrating peaks function of the gel densitometry software, Sigma-Gel

(Jandell Scientific, Sausalito, CA) to produce a profile (Fig. 5.5) and calculate peak areas.



**Figure 5.5** Profile produced following the densitometric analysis (see text) of protein bands in lane 8 of Fig. 5.4. Pixel intensity and migration distance are in relative units.

From Figure 5.5, the  $\alpha$  and  $\beta$  subunits were estimated to represent 63% of the purified  $CF_1$  sample, which was subsequently split into smaller aliquots to prevent any unnecessary cross contamination, and stored in liquid nitrogen until required.

### 5.3.2 *Quantification of standards and samples by electrophoresis, Western blotting and Enhanced Chemi-Luminescence (ECL)*

#### *Sample preparation*

All leaf samples for  $CF_1$  quantification had previously been harvested following gas exchange analysis (section 3.2), wrapped in foil envelopes, and stored in liquid nitrogen prior to analysis. With all subsequent operations done at 0 to 4°C,

leaf sections of approximately 300 mm<sup>2</sup> were removed from individual samples, cut into narrow strips approximately 1 mm wide, and ground in a pestle and mortar to which 1 mL of a 50 mM Bicine buffer (pH 7.6) containing 20 mM EDTA, 1 mM MgCl<sub>2</sub> and 50 mM  $\beta$ -Mercaptoethanol was added. This buffer had been optimised and checked for its ability to remove CF<sub>1</sub> from thylakoid membranes (see 5.3.3 below). Part of the homogenate was removed for the determination of Chl and total soluble protein content (sections 2.4 & 2.5) and then each sample was clarified at 10000 xg for 3 min. Supernatants were transferred to microcentrifuge tubes, a sub-sample taken to determine soluble protein content, and then kept on ice prior to SDS solubilisation.

### *Electrophoresis*

Samples and purified CF<sub>1</sub> standard (section 5.3.1) were solubilised on a SDS:protein basis of 4:1, in a 250 mM Tris buffer (pH 7.6) containing 250 mM Dithiothreitol, 10% (w/v) SDS, 0.2% (w/v) bromophenol blue and 10% (w/v) glycerol and incubated at 90°C for five minutes. If samples were not to be electrophoresed immediately, they were stored at -80°C.

Fifteen well SDS-polyacrylamide gels with a 12% discontinuous gradient (Bio-Rad Minigel) were used, and on each, four wells were loaded with CF<sub>1</sub> standard of either 0.1, 0.25, 0.35, 0.45 or 0.6  $\mu$ g, and a further well with a mixture of prestained molecular weight markers (Bio-Rad, Cat No 161-0305). Remaining wells contained 5  $\mu$ g loadings of samples, this being sufficient that the amount of CF<sub>1</sub> eventually resolved almost always fell within the standard range. Where this did not occur, samples were re-run as smaller loadings. Electrophoretic separation was at 130 V per two gels held within a Bio-Rad Mini-Gel system, for which both upper and lower reservoirs contained a Tris/SDS/glycine running buffer (Bio-Rad), which prior to use

had been diluted ten fold with distilled H<sub>2</sub>O in accordance with manufacturers instructions, and stored overnight at 0 to 4°C. Separation was normally complete after an hour when the dye front was within 5 mm from the bottom of gels. Gels were removed from the apparatus and their casing, and prior to Western blotting, were soaked for 15 minutes in chilled transfer buffer containing 25mM Tris, 192mM glycine pH 8.3 and 20% (v/v) methanol.

### *Western Blotting*

For each gel whilst they were soaking in transfer buffer, a piece of PVDF membrane (Millipore, Bedford, UK) was cut to 100 x 100 mm, pre-activated for 15 seconds in 100% methanol, rinsed for 5 minutes in distilled H<sub>2</sub>O and then pre-soaked in chilled transfer buffer for 15 minutes. During this time four pieces of Whatman 3MM filter paper were cut slightly larger than the PVDF membrane and also soaked in transfer buffer along with two fibre pads from the blotting cassette (Bio-Rad Trans Blot cell). Under transfer buffer, the cassette was assembled in the order; cassette holder, fibre pad, two sheets of Whatman 3MM, gel, PVDF membrane, two sheets of Whatman 3MM, fibre pad and cassette holder. To ensure a good contact between the gel and PVDF membrane, the membrane was smoothed onto the gel with a glass rod to exclude all air bubbles. The gel holder cassette was closed and inserted alongside that of the other cassette (two gels were usually processed at a time) into the buffer tank which was half full of transfer buffer. Cassettes were orientated such that gels were toward the cathode and PVDF membranes toward the anode. The tank was topped up with more transfer buffer, and electrophoretic transfer allowed to proceed at 1.8 mA 100 mm<sup>2</sup> for 2 hours. This length of time was sufficient to allow transfer onto the PVDF membrane, of all the dye from the pre-stained molecular weight

markers within the gel. Upon disassembly of cassettes, individual PVDF membranes were washed twice for five minutes each in 100 mL of a ten times phosphate buffered saline (10xPBS) solution containing 0.58M  $\text{Na}_2\text{HPO}_4$ , 170 mM  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  and 0.68M NaCl. The PVDF membrane was then placed in 100 mL of 1x PBS (which has a pH of 7.3 to 7.4), where it could remain at 4°C for up to three days prior to probing for the antigen of interest.

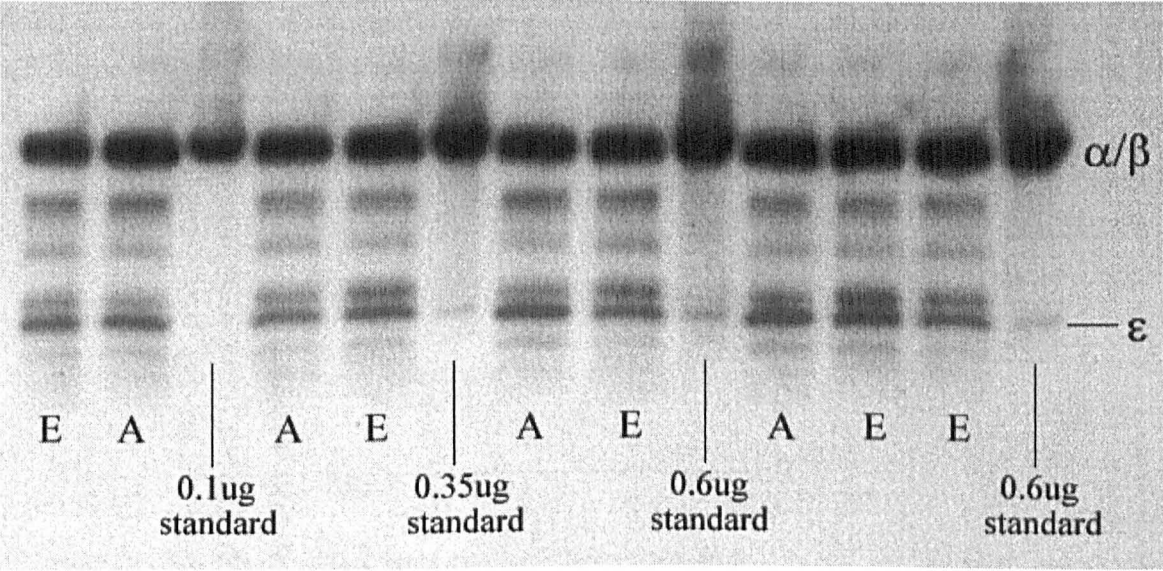
#### *Antigen detection and visualisation*

The antigen was visualised using an "Aurora" Western Blot Chemiluminescent Detection System (ICN Biomedicals, Costa Mesa, CA) for which the manufacturers protocol was followed. Briefly, individual PVDF membranes were blocked for an hour at room temperature on an orbital shaker in 100 mL of a blocking buffer which contained 0.2% (w/v) Aurora Blocking reagent, 10% (v/v) 1x PBS and 0.1% (v/v) Tween-20.

This was followed by a second incubation for an hour with 10 mL of blocking buffer which additionally contained primary antibody ( $\text{Ab}^1$ ) against the antigen of interest. In this instance  $\text{Ab}^1$ , raised in New Zealand white rabbits, was a kind gift from Professor J.C. Gray (University of Cambridge) and was specific against the alpha, beta and epsilon subunits of wheat  $\text{CF}_1$  (Howe et al., 1982). For use,  $\text{Ab}^1$  was diluted 4000 fold in blocking buffer as determined by an earlier dot blot experiment. Following this step, the membrane was washed twice for five minutes each in 50 mL of blocking buffer, and then incubated at room temperature for a further hour in 10 mL of blocking buffer containing a 10000 dilution of secondary antibody-alkaline phosphatase conjugate ( $\text{Ab}^2$ ) against rabbit  $\text{Ab}^1$ , which was supplied by the manufacturer. This  $\text{Ab}^2$  was washed from the membrane with three washes each of

five minutes in 50 mL of blocking buffer, before proceeding to chemiluminescent detection.

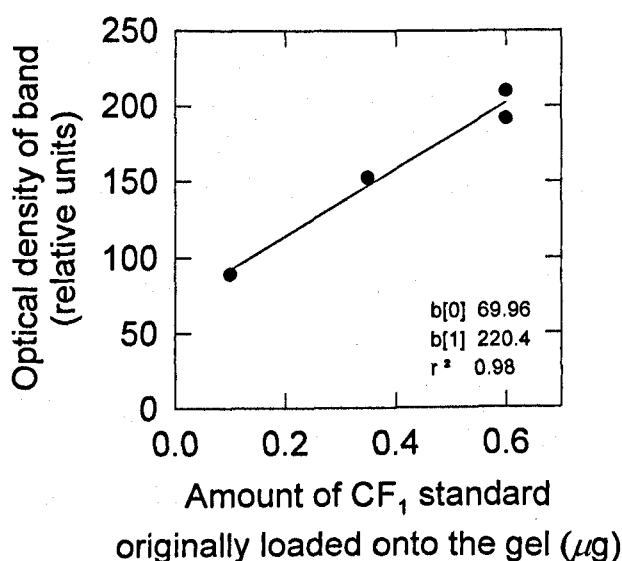
The membrane was washed twice for five minutes in 50 mL of an assay buffer which contained 0.1M Diethanolamine and 1mM MgCl<sub>2</sub> and was adjusted to pH 10.0 with HCl. This was followed by incubation at room temperature in 5 mL of assay buffer which additionally contained 50  $\mu$ L of “Starlight™” substrate.



**Figure 5.6** A typical 30 second exposure autoradiograph, obtained from a Western Blot for quantifying the  $\alpha$  and  $\beta$  subunits of the thylakoid ATP-ase in wheat. Samples are denoted by A for 36 Pa CO<sub>2</sub> grown plants and E for 70 Pa CO<sub>2</sub> grown plants. For all samples, 5 $\mu$ g of protein was loaded to each lane, and all were from leaves which had recently reached full expansion. Amounts of standard loaded on lanes are as indicated.

After five minutes, excess solution was drained away, and the membrane placed inside a clear plastic disposable wallet (supplied with the kit), and immediately exposed by direct contact for 30 seconds against standard Kodak XAR x-ray film, which in turn was developed immediately by standard procedure. Autoradiographs were scanned and the images saved as a TIF files (Eagle-Eye, Stratagene), Figure 5.6 being a typical example. Optical densities (O.Ds) of individual bands from these images corresponding to the position of the alpha and beta subunits of CF<sub>1</sub> were

quantified by following the manufacturers instructions of a computer software package (SigmaGel, Jandel Scientific), and then amounts determined for samples by extrapolation from a calibration curve made with these standards. The standard curve for the autoradiograph shown in Figure 5.6, is shown below in Figure 5.7. The total amount of CF<sub>1</sub> in samples was then calculated correcting for the fact that the alpha and beta subunits represented 63% of the total protein present in the purified CF<sub>1</sub> standard being used, and that in turn, the alpha and beta subunits constitute 81% of CF<sub>1</sub> (Moase and Green, 1981).



**Figure 5.7** Calibration curve resulting from the quantification of bands corresponding to the  $\alpha$  and  $\beta$  subunits of CF<sub>1</sub> standard loaded into lanes as indicated in the image of the autoradiograph in Fig. 5.6 above. The line is from a first order linear regression.

### *Results and Discussion*

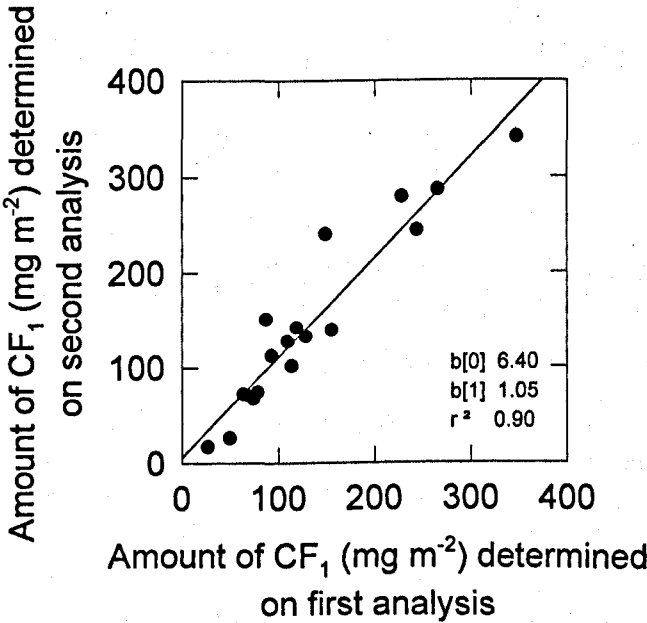
The amount of CF<sub>1</sub> in the ambient (A) samples shown in Figure 5.6, and estimated using the standard curve in Figure 5.7, are shown below in Table 5.11. The mean value of 0.41g CF<sub>1</sub> g Chl<sup>-1</sup> (Table 5.11, column five) obtained for these 36 Pa CO<sub>2</sub> grown samples, is in close agreement with that of 0.45g CF<sub>1</sub> g Chl<sup>-1</sup> suggested by Lawlor (1993, Table 4.1) for an 'average' C3 leaf and with that of 0.42g



CF<sub>1</sub> g Chl<sup>-1</sup> obtained in spinach (Strotmann et al., 1973) and within the range of 0.37 to 0.45g CF<sub>1</sub> g Chl<sup>-1</sup> of Frasch et al., (1980) also from spinach.

Lane in Fig 5.6	g m <sup>-2</sup>			g CF <sub>1</sub> g Chl <sup>-1</sup>
	Chl	Soluble Protein	CF <sub>1</sub>	
2	0.35	4.14	0.13	0.39
4	0.39	4.57	0.15	0.40
7	0.34	4.00	0.16	0.47
10	0.44	5.15	0.16	0.36
Mean	0.38 (±0.02)	4.47 (±0.26)	0.15 (±0.01)	0.41 (±0.02)

**Table 5.11.** The amounts of chlorophyll, soluble protein and CF<sub>1</sub> estimated using the method described in the text, for 36 Pa CO<sub>2</sub> grown flag leaves of wheat at full emergence, and shown as samples (A) in figure 5.6 above. The value in brackets for the mean of g CF<sub>1</sub> g Chl<sup>-1</sup> is 1x Std Err.



**Figure 5.8** Correlation between the amount of CF<sub>1</sub> in the same individual SDS solubilised samples, but analysed on different days, in different gels and subject to the inherent variation in electrophoretic separation and transfer etc. Data points are from flag-1 leaf samples of both the low and high N treatment of Experiment Two. The line is a first order linear regression.

The general robustness of the assay was confirmed by the good correlation observed for the amount of CF<sub>1</sub> measured in the same individual SDS solubilised extracts, but on different days and in different gels (Fig. 5.8).

Regarding autoradiographs (e.g. that illustrated in Figure 5.6), it is clear that apart from the  $\alpha$ ,  $\beta$  and  $\epsilon$  subunits, there was some cross reaction with other bands in lanes containing samples, despite the statement in the paper of Howe et al., (1982) which describes the preparation of the antiserum against  $CF_1$  used here, stating that there was “no indication of any cross-reaction with other subunits” apart from those to which the antiserum was prepared. In section 5.3.3 and Figure 5.9 which accompanies it, it is demonstrated that  $Ab_1$  against  $CF_1$  has no cross reaction with either the LSU or SSU of Rubisco, so these possibilities can be eliminated. From comparing the relative positions of bands in Figure 5.6 with peaks in Figure 5.5, the band immediately beneath the  $\alpha$  and  $\beta$  subunits is most likely to represent the  $\gamma$  subunit, and the band beneath this the  $\delta$  subunit of  $CF_1$ . Given that total leaf extracts were run on gels, it is possible that the band immediately above, but still comparatively close to the  $\epsilon$  subunit, is also perhaps an  $\epsilon$  subunit, but derived from mitochondrial ATP-synthase.

### 5.3.3 *Experiments to determine whether $CF_1$ and Rubisco could be accurately quantified from a single extraction of each leaf sample*

#### *Experiment One*

##### *Introduction*

For reasons of accuracy, in experiments where the aim has been to quantify various leaf components and relate them to each other, this has usually been achieved by analysing the components of interest in individual aliquots of a single leaf extraction (Evans, 1983; Makino et al., 1997). Given that one of the aims of this study was to understand relationships between the amounts of Rubisco and  $CF_1$

within individual leaves, a similar single extraction approach was sought here. However, Rubisco and CF<sub>1</sub> have differing buffer requirements for successful extraction. Typically, Rubisco requires a high cation concentration (e.g. 20mM Mg<sup>2+</sup>), whilst conversely, CF<sub>1</sub> requires a low cation concentration for its removal from membranes, for which the chelator EDTA is typically employed at concentrations of 20mM (Lawlor, 1993). Thus the aim of this experiment was to use buffers optimised for either Rubisco or CF<sub>1</sub> extraction, and determine how the recovery of the alternative component of interest was affected.

### *Materials and Method*

In a cold room at 0 to 4°C, 240mm<sup>2</sup> of young fully expanded wheat leaf material was cut into 1 mm wide strips, mixed thoroughly, and divided into two subsamples on a FW basis. In a pestle and mortar on ice, one subsample was homogenised in 2 mL of a 50 mM Bicine extraction buffer (pH 7.6), containing 20 mM MgCl<sub>2</sub>, 1 mM EDTA and 50 mM  $\beta$ -Mercaptoethanol. This was the standard buffer used for extracting Rubisco (section 4.2.3), and hereafter is referred to as buffer A. The extract was removed into a microcentrifuge tube, and the mortar rinsed with a further 0.5 mL of buffer A, and this was combined and mixed with the first. From this homogenate, two 50  $\mu$ L aliquots were removed for the determination of chlorophyll content (section 2.4). The homogenate was clarified at 12000 xg for 3 minutes and the supernatant retained, from which further aliquots were removed for the determination of total soluble protein content. The second subsample was processed in exactly the same way, but for the use of buffer B optimised for CF<sub>1</sub> removal, which was also a 50 mM Bicine extraction buffer (pH

7.6) with 50 mM  $\beta$ -Mercaptoethanol, but containing 1 mM  $\text{MgCl}_2$  and 20 mM EDTA.

With the appropriate buffer, the two samples were diluted on a soluble protein basis to 1  $\mu\text{g}/\mu\text{L}$ , and then solubilised on a SDS to protein basis of 4:1, using the solubilisation buffer described in section 5.3.2. These samples were then electrophoresed, blotted and visualised using the  $\text{CF}_1$  quantification method described in 5.3.2.

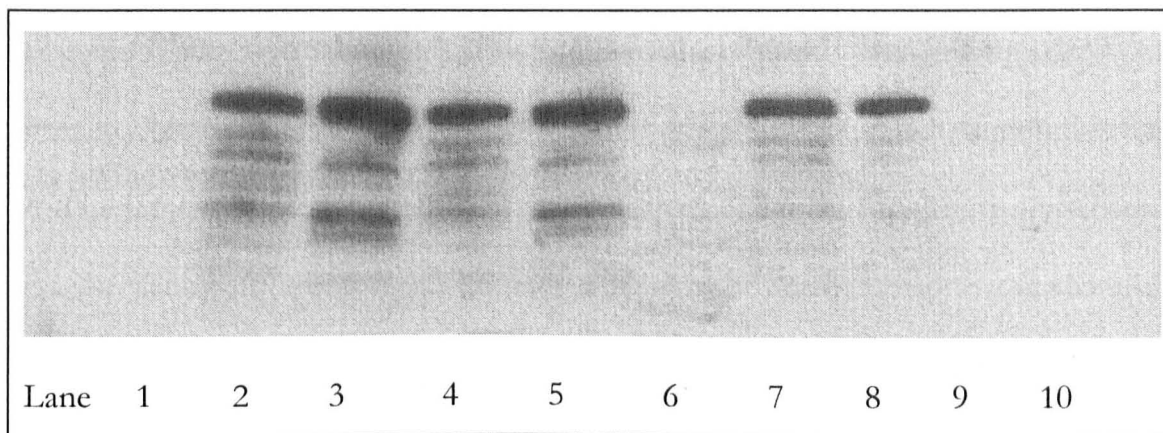
### *Results and Discussion*

The concentration of chlorophyll in both subsamples was not significantly different, whilst the concentration of soluble protein was significantly different ( $P < 0.05$ ), being greatest in the subsample where buffer B was used during extraction (Table 5.12 below).

	Buffer 'A'	Buffer 'B'
Chlorophyll ( $\text{g m}^{-2}$ )	0.79 ( $\pm 0.054$ ) <sup>a</sup>	0.77 ( $\pm 0.023$ ) <sup>a</sup>
Soluble Protein ( $\text{g m}^{-2}$ )	6.83 ( $\pm 0.043$ ) <sup>a</sup>	7.18 ( $\pm 0.035$ ) <sup>b</sup>

**Table 5.12** A comparison between the amounts of Chlorophyll and soluble protein measured in two identical subsamples extracted in either buffer A optimised for the removal of Rubisco, or buffer B optimised for the removal of  $\text{CF}_1$ . Values are the mean of three independent estimations  $\pm$  SE; different superscript characters indicate statistical differences between columns at  $P \leq 0.05$ .

The resultant TIF file image of the autoradiograph from the experiment is shown below (Fig 5.9).



**Figure 5.9** Autoradiograph showing the response of antiserum against the  $\alpha$  &  $\beta$  subunits of  $CF_1$  in an experiment to determine the potential for using a single leaf extraction for quantifying Rubisco and  $CF_1$ . Lanes 1 & 10 contain molecular weight markers, lanes 2 & 4 contain 10 and 5  $\mu g$  respectively of sub-sample extracted using Buffer A optimised for the removal of Rubisco, lanes 3 & 5 contain 10 and 5  $\mu g$  respectively of sub-sample extracted using Buffer B optimised for the removal of  $CF_1$ , lanes 6 & 9 contain 10 and 5  $\mu g$  loadings of Rubisco standard, and lanes 7 & 8 contain repeat runs of a sample from a previous experiment not discussed further. For further details see text.

Lanes two and three contained 10  $\mu g$  soluble protein loadings of subsamples one and two respectively, and it is clear from this that subsample two (extracted with buffer B optimised for  $CF_1$  removal), contained proportionately more  $CF_1$ , despite the equal loadings, and the fact that the subsamples were identical based on their similar chlorophyll contents (Table 5.12). Lanes four and five, in which 5  $\mu g$  of soluble protein were loaded to each, verify the result, so suggesting that if buffer A (optimised for Rubisco) were to be used for all extractions, the amount of  $CF_1$  could be underestimated by as much as 20%, based on measured O.D differences between the two subsamples. There was no cross-reaction between antiserum against  $CF_1$  and either the SSU or LSU of Rubisco standard in lanes six and nine (Fig. 5.9).

## *Experiment Two*

### *Introduction*

To perform the reciprocal analysis, and determine how the choice of extraction buffer affected the recovery of Rubisco, the PVDF membrane from experiment one was stripped according to the manufacturers protocol (ICN Biomedicals), and reprobed with the same Ab<sub>1</sub> against Rubisco previously used in the ELISA assay (section 4.3).

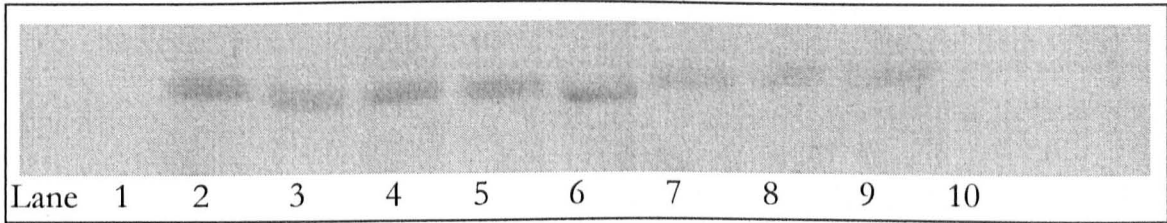
### *Materials and Method*

Briefly, at room temperature, the previous detection antibodies and chemiluminescent substrate from experiment one were removed by washing the membrane for 60 minutes in 150 mL of stripping buffer containing 0.2 M Glycine (pH 2.2 with HCl), 0.1% (v/v) SDS and 1% (v/v) Tween-20. This was followed by three successive five minute washes in 150 mL of wash buffer consisting of 0.1% (v/v) Tween-20 in 1xPBS. The Aurora Western blot protocol (ICN Biomedicals) described in 5.3.2 was then followed beginning with the incubation in blocking buffer, and the subsequent modifications that monoclonal Ab<sub>1</sub> against the LSU of Rubisco which had been raised in rat was diluted 100-fold, and Ab<sub>2</sub> specific against rat antisera then followed.

### *Results and Discussion*

For an unknown reason, final exposure of the developed PVDF membrane to film for 20 minutes revealed no image (30 s was usually sufficient), so a second film was left to expose overnight, which revealed the faint banding shown in Figure 5.10 below. Unfortunately the intensity of bands were insufficient for accurate

quantification by the SigmaGel software, but nevertheless provided useful confirmation that Ab<sub>1</sub> against Rubisco bound to the LSU. It seems most probable that the explanation for the poor overall intensity of bands obtained on this second image, lies with the stripping of the previously applied antibodies and chemiluminescent substrate, which may in this instance, have additionally removed antigen as well. It seems unlikely however, that the faint banding was due to a lack of Ab<sub>1</sub>, since here Ab<sub>1</sub> was less dilute at 100-fold dilution, than in the 1000-fold dilution used in the ELISA assay (4.3.2).



**Figure 5.10** Autoradiograph showing the response of antisera against the LSU of Rubisco in an experiment to determine the potential for using a single leaf extraction for quantifying Rubisco and CF<sub>1</sub>. Lanes 1 & 10 contain molecular weight markers, lanes 2 & 4 contain 10 and 5  $\mu$ g respectively of sub-sample extracted using Buffer A optimised for the removal of Rubisco, lanes 3 & 5 contain 10 and 5  $\mu$ g respectively of sub-sample extracted using Buffer B optimised for the removal of CF<sub>1</sub>, lanes 6 & 9 contain 10 and 5  $\mu$ g loadings of Rubisco standard, and lanes 7 & 8 contain repeat runs of a sample from a previous experiment not discussed further. For further details see text.

### *Experiment Three*

The experiment was repeated starting with fresh leaf material, extracting two subsamples with buffers A and B as before, but this time quantifying Rubisco using the <sup>14</sup>CABP assay described in 4.4. This was done, and the results shown in Table 5.13 below were obtained.

## Results and Discussion

As in the previous experiment, there was no significant difference between the two subsamples in terms of their chlorophyll contents, but soluble protein content was significantly ( $P<0.01$ ) increased by 16% in the subsample extracted with buffer B (Table 5.13).

	Buffer 'A'	Buffer 'B'
Chlorophyll ( $\text{g m}^{-2}$ )	0.75 ( $\pm 0.012$ ) <sup>a</sup>	0.84 ( $\pm 0.095$ ) <sup>a</sup>
Soluble Protein ( $\text{g m}^{-2}$ )	6.98 ( $\pm 0.173$ ) <sup>a</sup>	8.09 ( $\pm 0.092$ ) <sup>b</sup>
Rubisco ( $\text{g m}^{-2}$ )	2.96 ( $\pm 0.009$ ) <sup>a</sup>	2.49 ( $\pm 0.019$ ) <sup>b</sup>

**Table 5.13** A comparison between the amounts of Chlorophyll, soluble protein and Rubisco measured in two identical subsamples extracted in either buffer A optimised for the removal of Rubisco, or buffer B optimised for the removal of  $\text{CF}_1$ . Values are the mean of three independent estimations  $\pm$  Std Err; different superscript characters indicate statistical differences between columns at  $P\leq 0.05$ .

However, the buffer A extracted subsample which had less soluble protein, had the significantly ( $P<0.001$ ) greater (19% more) Rubisco content.

Clearly the combined results of these two experiments demonstrate that the use of a single extraction buffer which may be optimised for one particular component (in this case Rubisco or  $\text{CF}_1$ ), will always result in an underestimation of the amount of the other component of interest, and thus it was clearly impractical to use a single leaf extraction to determine accurately both Rubisco and  $\text{CF}_1$ , to the degree of accuracy required in this study.



## 5.4 The effect of long-term growth at 70 Pa CO<sub>2</sub> on the amount of coupling factor (CF<sub>1</sub>)

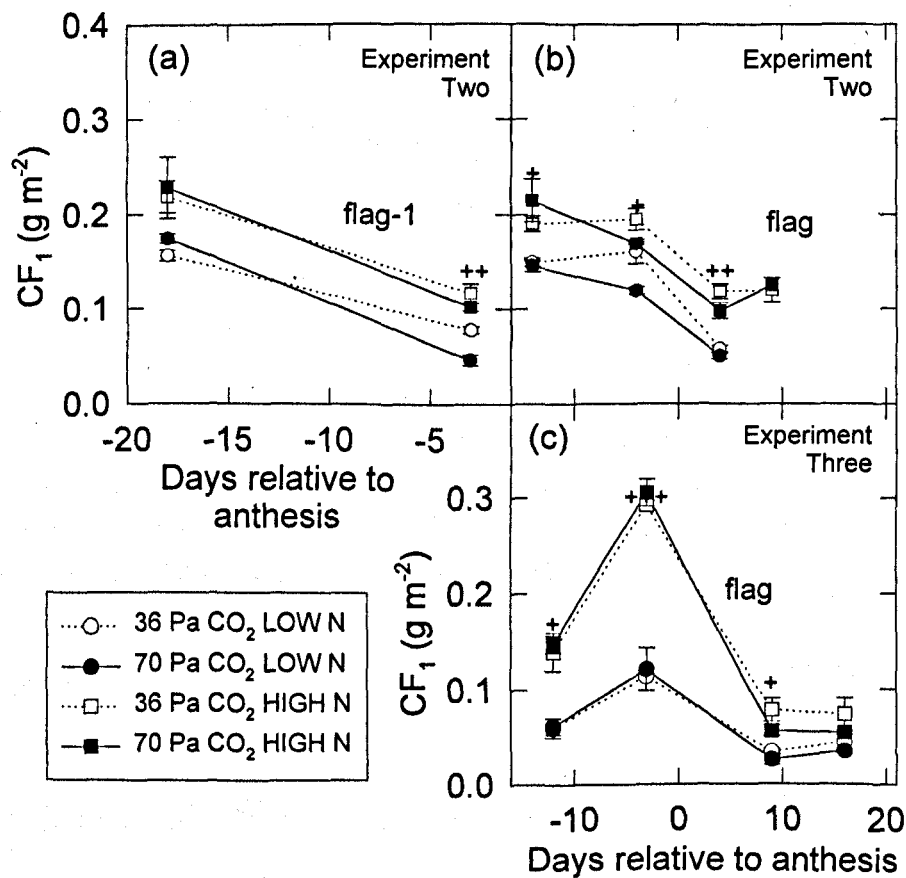
### 5.4.1 *Introduction*

To determine whether or not long-term growth at elevated CO<sub>2</sub> would affect the amount of the CF<sub>1</sub> component of the thylakoid ATP-ase in leaves of spring wheat, the SDS-PAGE/Western blot/ECL technique described in 5.3.2 above, was applied to samples collected from two separate controlled environment experiments, on plants grown as crop stands. The details of these experiments are described fully in Chapter Two, however briefly, for Experiment Two, plants were grown with total N applications of 7 (low) or 19 (high) g N m<sup>-2</sup>, and in Experiment Three at either 8 (low) or 21 (high) g N m<sup>-2</sup>, and for both experiments, at either 36 or 70 Pa CO<sub>2</sub>. From tillering to maturity plants were grown with day/night temperature regimes of 20/7°C, and a constant artificial PPFD of 580  $\mu$ mol quanta m<sup>-2</sup> s<sup>-1</sup> at plant height. Leaf samples for analysis (which correspond to those also used for gas exchange analysis as discussed in Chapter Three), were harvested from full leaf emergence, through grain-filling and into senescence, at approximately ten day intervals. Results are presented and discussed, first as a function of leaf area content through time, and then as a function of leaf area content versus total leaf N.

### 5.4.2 *Results - amount of CF<sub>1</sub> in leaves as a function of time*

In all leaves of both experiments, absolute values for CF<sub>1</sub> within a sampling occasion were always greater in plants grown at the higher N application compared to low (Fig. 5.11), and these differences were always significant ( $P < 0.05$ ), except for

the first occasion (full emergence) of flag-1 in Experiment Two, and the final occasion of the flag leaf in Experiment Three (Fig. 5.11 a & c respectively).



**Figure 5.11** The response through time, as days relative to anthesis, of the amount of  $CF_1$  measured in flag-1 (a) and flag leaves (b & c) of wheat from Experiments Two (a & b) and Three (c), grown at  $CO_2$  and N applications as indicated in the key. Each point represents the mean of three samples, and where visible error bars = 1 x Std Err. Plus signs denote levels of significance from  $CO_2 \times N$  ANOVA (Microsoft Excel) for each occasion, where + =  $P < 0.05$ , ++ =  $P < 0.01$  and +++ =  $P < 0.001$ . For further details see text.

However, the levels of significance for Experiment Three, with the exception of the second occasion, were much lower than they had been for either Rubisco or leaf N content against time (Figs 4.6 d & 3.3 d), implying that there is more variability in  $CF_1$  values than Rubisco and N for Experiment Three.

For all leaves of both experiments and on all occasions, there was no significant effect of the  $CO_2$  treatment on the amount of  $CF_1$ . However, despite this,

from three days before anthesis for both leaves of Experiment Two (Fig. 5.11 a & b), and from nine days post-anthesis in Experiment Three (Fig. 5.11 c), amounts of CF<sub>1</sub> in 70 Pa CO<sub>2</sub> grown leaves were always slightly lower than in 36 Pa CO<sub>2</sub> grown, for a given N treatment. The exception to this is the fourth occasion, high N treatment of the flag leaf in Experiment Two.

There was no interaction of N and CO<sub>2</sub>, in any leaf of any occasion or experiment. Data for the second occasion (10 days before anthesis) of flag-1 of Experiment Two (Fig. 5.11 a) are not shown, since the estimated CF<sub>1</sub> values as a fraction of soluble protein content, were abnormally high for all treatments (>4.4%).

The CF<sub>1</sub> analysis for both leaves of Experiment Two, had been done on the remaining halves of individual leaves (hereafter referred to as CF<sub>1</sub> leaves) for which the other half had already been used to quantify Rubisco (hereafter referred to as Rubisco leaves), the results of which have been presented and discussed previously in Chapter Four, and to which the results here for CF<sub>1</sub> are directly comparable. In connection with this, the data for the amount of leaf N as a function of time, is also the same as shown in Figure 3.3 b & c, and therefore is not repeated again here.

For each treatment and occasion in Experiment Three, six replicate leaf samples had been measured for gas exchange analysis (section 3.2); three of these leaf samples were each individually divided in two, one half being used to determine Rubisco content and the other half total leaf N. The three remaining leaf samples have been used here to determine CF<sub>1</sub> content (CF<sub>1</sub> leaves). Therefore, as in Experiment Two, here the graph for leaf N as a function of time relating to CF<sub>1</sub> leaves is the same as that shown in Figure 4.6 relating to Rubisco leaves, and so has not been shown again.

### 5.4.3 Discussion

As in the response of Rubisco and leaf N content against time (discussed in 4.6.3), the general decline (regardless of specific treatments) in absolute values for CF<sub>1</sub> content, reflects a natural ontogenic decline of components and resources within leaves as they are remobilised for the developing ear and grain. Therefore, it is of little surprise that leaves which contain lesser amounts of components to begin with at full emergence, senesce earlier as is clearly seen in this data (Fig. 5.11) for CF<sub>1</sub> content.

The effect of the two N treatments on the amount of CF<sub>1</sub> in leaves of Experiment Three (Fig. 5.11 c), is largely explained by effects on leaf N content against time (Fig. 3.3 d).

That there was no significant effect of CO<sub>2</sub> treatment on the amount of CF<sub>1</sub> in flag or flag-1 leaves at full expansion (occasion one; Fig. 5.11 a-c) in either experiment, is consistent with the findings of Nie et al., (1995a), for the same component in the fifth, eighth and flag leaves of spring wheat at full emergence, and grown at 36 or 55 Pa CO<sub>2</sub> in a FACE experiment. Further, in this current study, there was no evidence for any subsequent effect of CO<sub>2</sub> treatment on the amount of CF<sub>1</sub>, and this is also in agreement with the findings of Nie et al., (1995a).

However, despite the lack of any significant CO<sub>2</sub> effect, there were many occasions within a given N treatment, both in leaves of this experiment and on later occasions in those reported in Nie et al., (1995a), when the amount of CF<sub>1</sub> per unit leaf area was less in leaves of elevated CO<sub>2</sub> grown plants, compared to ambient. This observation would seem contrary to the hypotheses being tested in this chapter, that the amount of CF<sub>1</sub> should increase in leaves at elevated CO<sub>2</sub> to rebalance capacities. However, elevated CO<sub>2</sub> can induce a general decrease in leaf N content. On some

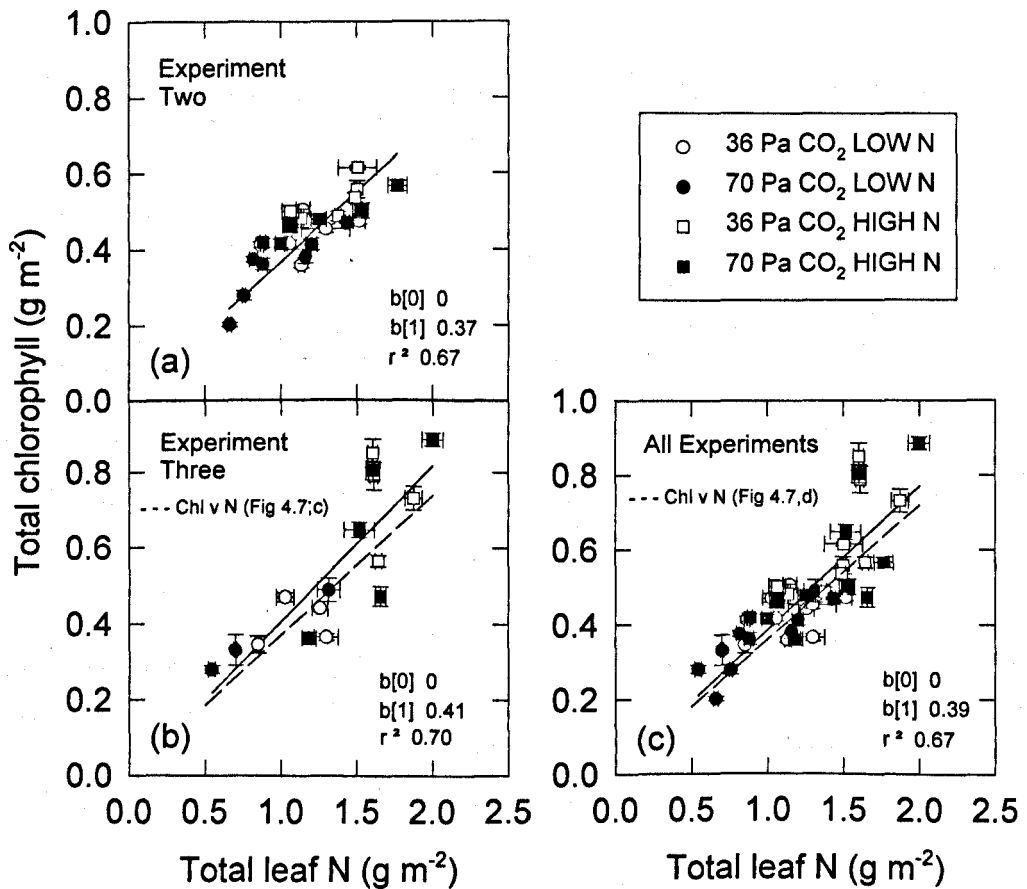
occasions, it is arguable that the content of leaf N in 70 Pa CO<sub>2</sub> grown leaves, decreased significantly faster than CF<sub>1</sub> content (e.g. Experiment Three, low N treatment on occasions three and four, compare Fig. 5.11 d with 3.3 d), which could be consistent with a re-balancing of capacities. Thus, in order to determine whether the amount of CF<sub>1</sub> as a proportion of total leaf N had or had not been affected by growth at 70 Pa CO<sub>2</sub>, it was necessary to replot data on this basis.

#### 5.4.4 *Results - amounts of Chl, soluble protein and CF<sub>1</sub> as a proportion of total leaf N - effects of elevated CO<sub>2</sub>*

Data in the following section has been analysed by fitting regressions by the method of least squares (Microsoft Excel) to individual treatments for each relationship, and making comparisons between regression line slopes within each factor. Where they occurred, differing sample sizes and heterogeneous variances have been accounted for. All calculations were made using the equations described in Kassab (1988), and computed t-values have been tested at  $\alpha = 0.05$ .

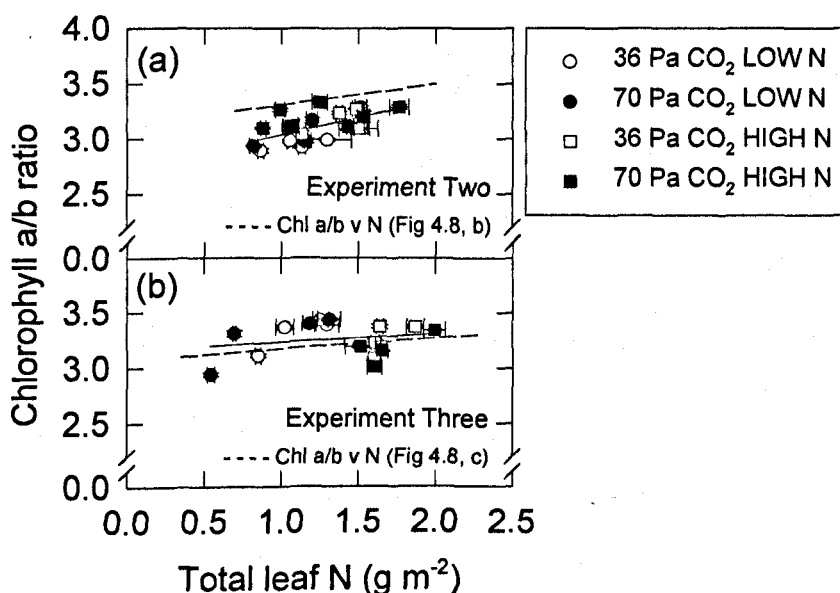
The total chlorophyll content measured in samples of CF<sub>1</sub> leaves, was approximately proportional to leaf N content, and this pattern was seen in all leaves of both experiments (Fig 5.12 a & b). For all, the slope of the relationship was independent of N and CO<sub>2</sub> treatment.

The regression for Experiment Two (Fig. 5.12 a) intercepted the y-axis at 0.15g Chl m<sup>-2</sup>, presumably due to the 30% narrower range of N contents within leaves that were sampled, whilst that for Experiment Three (Fig. 5.12 b) intercepted the x-axis very close to the origin (0.02g N m<sup>-2</sup>).



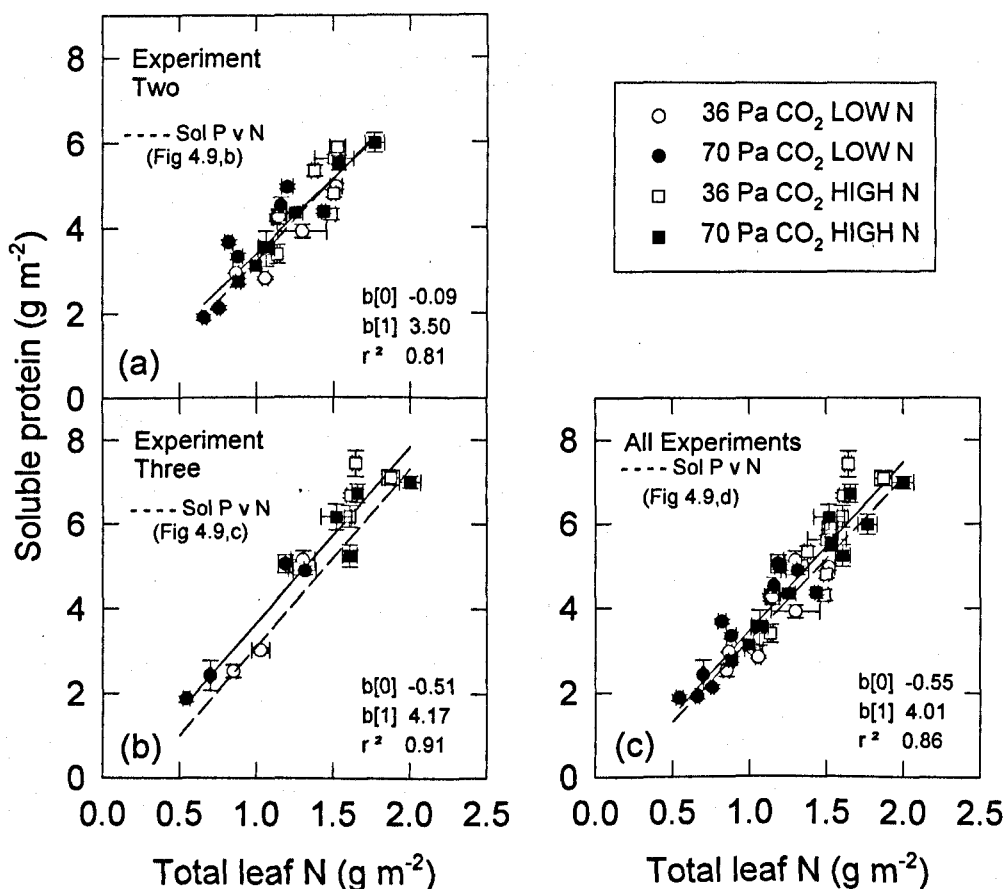
**Figure 5.12** Amounts of Chlorophyll versus total leaf N content, measured from full emergence through to senescence in a combination of flag-1 and flag leaves in Experiment Two (a), and flag leaves in Experiment Three (b). Experimental treatments are summarised in the key. Points for both experiments are the mean of three Chlorophyll versus the mean of three N values in leaves of the same occasion and treatment, with 1 x Std Err shown for both axis of each. All panels show first order linear regressions forced through the origin, with the resultant equation. The "All Experiments" panel (c) shows the combined data for both experiments. In panels (b) & (c), the dashed lines shown for comparison represent regressions for the same relationship, but obtained in equivalent leaf halves (Experiment Two) or equivalent leaves (Experiment Three) selected for Rubisco quantification as previously presented in Fig 4.7. No comparable regression is shown for panel (a), as both regressions were identical. For further details see text.

The slopes of the relationships shown here for CF<sub>1</sub> leaves of each experiment, are not significantly different from, the slopes for the same relationship, but in comparable Rubisco leaves (Fig. 4.8 a to d), the regressions for which have been plotted here (Fig 5.12 a to c) for comparison, but not distinguishable in Figure 5.12 a, as the positions of the regression lines are identical.



**Figure 5.13** The Chlorophyll a/b ratio versus total leaf N content measured in samples principally extracted for  $CF_1$  quantification, from flag-1 and flag leaves in Experiment Two, and flag leaves in Experiment Three, taken from full emergence through to senescence. Experimental treatments are as described in the key. Points for both experiments are the mean of three Chlorophyll a/b ratios versus the mean of three N values in leaves of the same occasion and treatment, with 1 x Std Err shown for both axis of each. Both panels show first order linear regressions. Dashed lines represent regressions obtained for the same relationship, but in equivalent leaf halves (Experiment Two) or leaf samples (Experiment Three), principally extracted to determine Rubisco content. For further details see text.

The slopes of the relationship between Chl a/b ratio and total leaf N content in all leaves of both experiments, were independent of CO<sub>2</sub> and N treatments during growth (Fig. 5.13 a & b). For the combined data of flag-1 and flag leaves of Experiment Two (Fig. 5.13 a), the ratio increased from 2.9 to 3.25 with a 1.05g m<sup>-2</sup> increase in leaf N content. This relationship was not significantly different from that for the same relationship in equivalent halves of Rubisco leaves for the same experiment obtained previously (Fig. 4.8 b). For Experiment Three (Fig. 5.13 b), the Chl a/b ratio was nearly constant as a function of leaf N, and also not significantly different from the slope for the same relationship, determined from equivalent Rubisco leaves (Fig. 4.8 c).



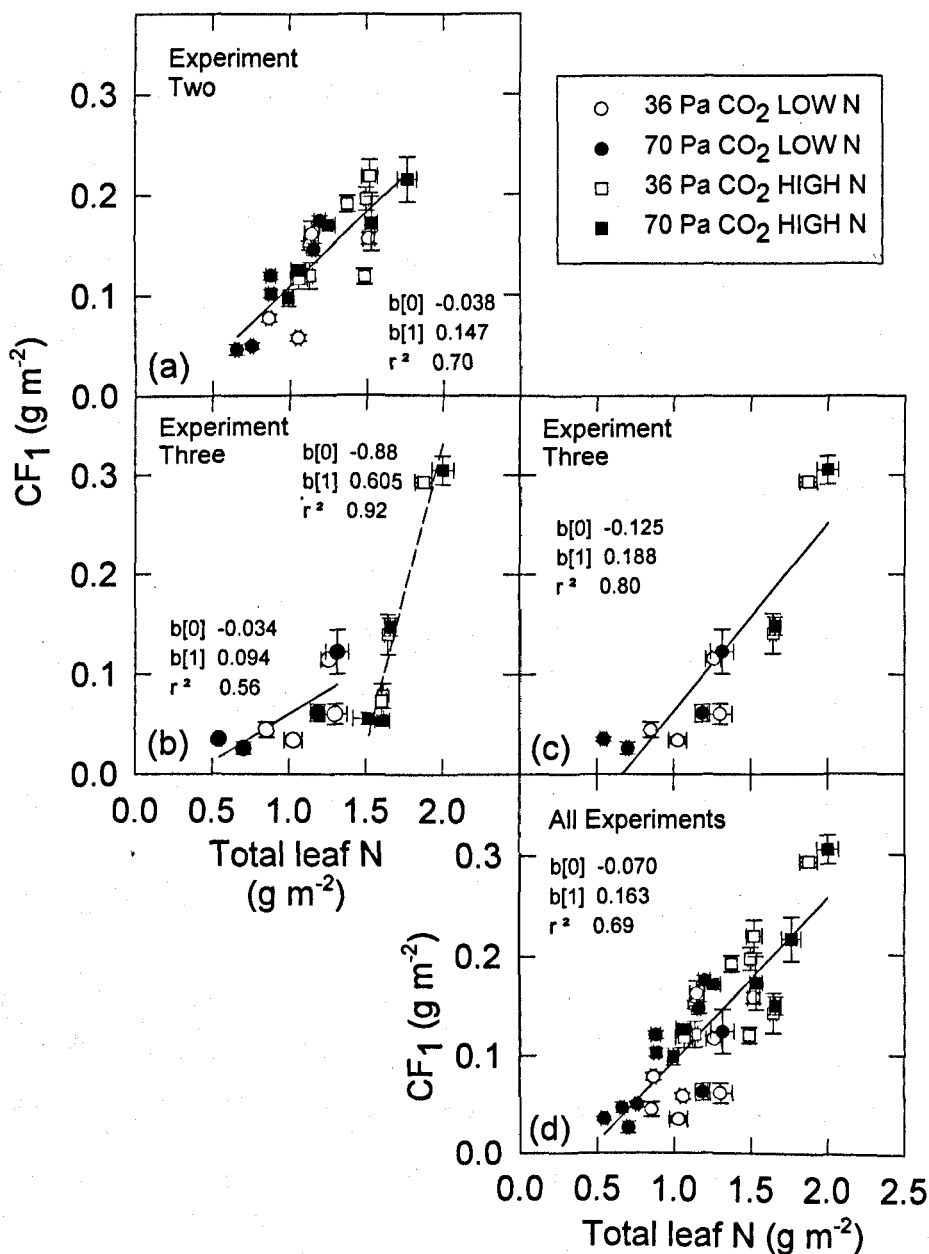
**Figure 5.14** Relationship between soluble protein and total leaf N content, measured from full emergence through to senescence in a combination of flag-1 and flag leaves in Experiment Two (a), and flag leaves in Experiment Three (b). Experimental treatments are summarised in the key. Points for both experiments are the mean of three soluble protein versus the mean of three N values in leaves of the same occasion and treatment, with 1 x Std Err shown for both axis of each. All panels show first order linear regressions (solid lines) forced through the origin, with the resultant equation. The 'All Experiments' panel (c) shows the combined data for both experiments. For ease of comparison, the dashed lines in all panels, represent regressions for the same relationship, but obtained in equivalent leaf halves (experiment two) or equivalent leaves (experiment three) selected for Rubisco determination as previously presented in Fig 4.9. For further details see text.

For the relationship between soluble protein and total leaf N content (Fig. 5.14 a to c), there was again, no significant effect of CO<sub>2</sub> or N treatment on the slope of this regression, in any of the leaves in either experiment. Further, the slopes of the relationships in these CF<sub>1</sub> leaves, were not significantly different from those obtained in Rubisco leaves (Fig. 4.9 b & c), regressions for which are indicated in Figure 5.14 by dashed lines for ease of comparison.



The relationship between  $CF_1$  and total leaf N content, for the combined data of flag-1 and flag leaves from Experiment Two, is shown below (Fig. 5.15 a). Both N and  $CO_2$  had no significant effect on the slope of this relationship, which intercepted the x-axis at  $0.25\text{g N m}^{-2}$ , suggesting that as total leaf N content increased, so did the proportion of N invested into  $CF_1$ , implying that during leaf senescence,  $CF_1$  declines faster than N as a whole.

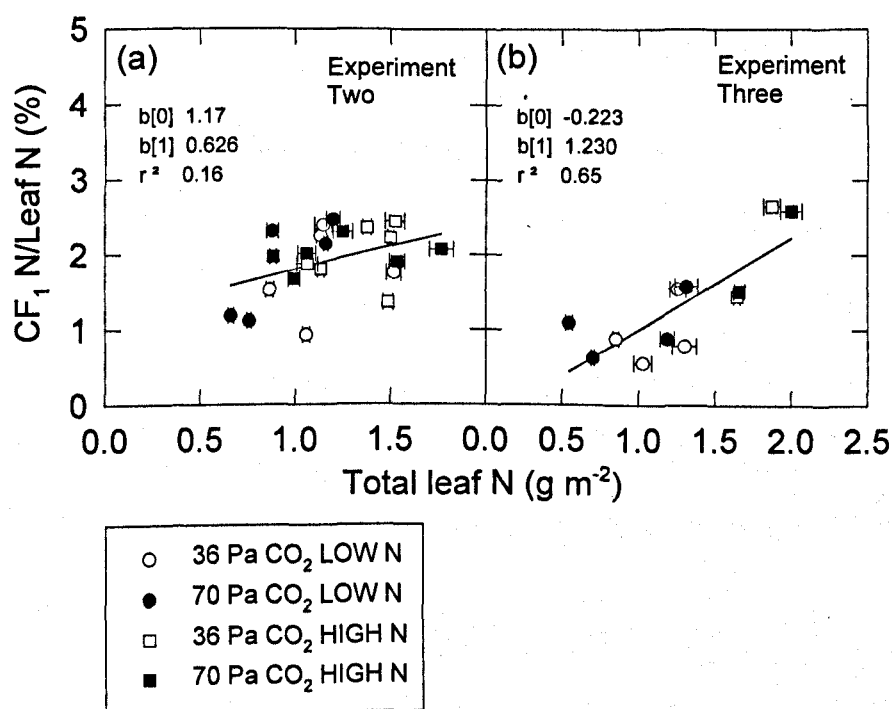
The same, but clearly less well defined relationship for the flag leaves of Experiment Three is shown in Figure 5.13 b. Here the slope of the relationship was independent of  $CO_2$ , but significantly ( $P < 0.05$ ) dependent on the N treatment applied during growth, as the two regression lines indicate. However, despite high leaf N contents, some of the points for the high N treatment had unusually low  $CF_1$  contents, all of which corresponded to leaves sampled on occasions three and four (as illustrated in Fig. 5.11 c). These same samples also correspond to those outlying in the relationship between  $CF_1$  and chlorophyll content (Fig. 5.17 and text below). Given this evidence, these data points were removed, and the data replotted to give the improved relationship shown in Figure 5.15 c. As for Experiment Two, the slope of the regression was independent of both  $CO_2$  and N, but here was slightly steeper, and there was an x-axis intercept at  $0.7\text{g N m}^{-2}$ . This suggested, as for Experiment Two, that as total leaf N content increased, so did the proportion of N invested into  $CF_1$ . This conclusion is supported by the data presented in Figure 5.16, which shows the change in the proportion of N allocated to  $CF_1$ , with increasing leaf N content. For both experiments, the proportion of N allocated to  $CF_1$  increased with increasing leaf N content, the relationships for both experiments being independent of the  $CO_2$  and N treatment applied during growth.



**Figure 5.15** The relationship between  $CF_1$  and total leaf N content measured from full emergence through to senescence in a combination of flag-1 and flag leaves in Experiment Two (a), and flag leaves in Experiment Three (b & c). Experimental treatments are summarised in the key. Points for both experiments are the mean of three  $CF_1$  versus the mean of three N values in leaves of the same occasion and treatment, with 1 x Std Err shown for both axis of each. All panels show first order linear regressions, with the resultant equation. The 'All Experiments' panel (d) shows the combined data for both experiments. For further details see text.

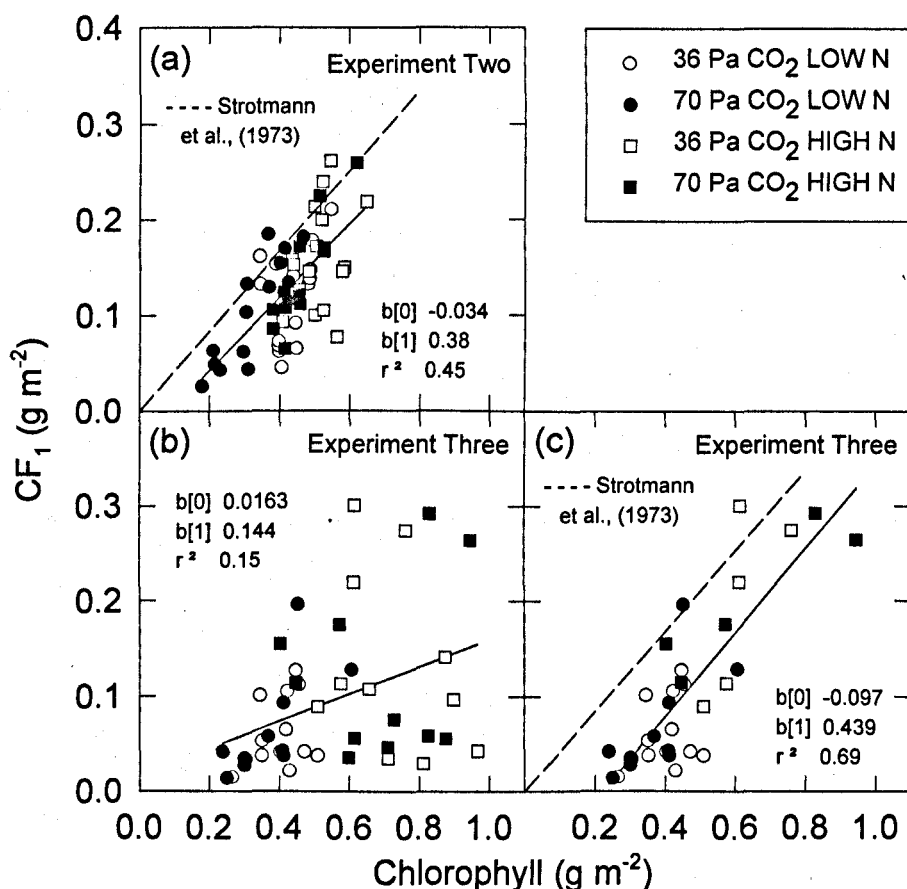
In Experiment Two (Fig. 5.16 a), the percentage of leaf N allocated to  $CF_1$  increased from 1.6 to 2.25%, with an increase in leaf N content from 0.7 to 1.7 g m<sup>-2</sup>, whilst in Experiment Three (Fig. 5.16 b), the percentage of leaf N allocated to  $CF_1$

increased from 0.7 to 1.85% over the same leaf N range, indicating that the relationships between the two experiments differed widely, results for Experiment Two being almost double those of Experiment Three.



**Figure 5.16** Coupling factor 1 N as a percentage of total leaf N versus total leaf N content measured in (a) samples of flag-1 and flag leaves (Experiment Two), and (b) flag leaves (Experiment Three), from full emergence through to senescence. Treatments are as indicated in the key. Each point plotted in (a) and (b), is the fraction of  $CF_1 N$  of total leaf N (where the  $CF_1$  value used was the mean of three, derived from Figure 5.10) versus the mean of three N values in leaves of the same occasion and treatment, with 1 x Std Err shown for the x-axis. Both panels show first order linear regressions and the resultant equation. For further details see text.

Lastly, the relationship between  $CF_1$  and chlorophyll content, for the combined data of flag-1 and flag leaves from Experiment Two, is shown below (Fig. 5.17 a).



**Figure 5.17** The relationship between  $CF_1$  and Chl content for samples taken from full emergence through to senescence, in a combination of flag-1 and flag leaves in Experiment Two (a), and flag leaves in Experiment Three (b). Panel (c) is (b) replotted, but with data for the high N treatment, occasions three and four removed. Dashed lines shown for comparison in panels (a) and (c), represent the relationship of  $0.42\text{g CF}_1 \text{ g}^{-1} \text{ Chl}$  obtained for spinach by Strotmann et al., (1973). Treatments are as indicated in the key. Solid lines in all panels represent first order linear regressions, with the resultant equation as shown. For further details see text.

Both N and  $\text{CO}_2$  had no significant effect on the slope of this relationship, which intercepted the x-axis at  $0.09\text{g Chl m}^{-2}$ . The same, but less clearly defined relationship for the flag leaves of Experiment Three, which had a y-axis intercept at  $0.02\text{g CF}_1 \text{ m}^{-2}$ , is shown in Figure 5.17 b. The slope of the relationship was independent of both  $\text{CO}_2$  and N treatments. However, as for the relationship between  $CF_1$  and leaf N content (Fig. 5.15 b), for this experiment, despite high leaf chlorophyll contents, some of the points for the high N treatment had unusually low amounts of  $CF_1$ . Again these corresponded to leaves sampled on occasions three and

four. Removing these points, improved the  $R^2$  considerably ( $R^2 = 0.69$ ), and resulted in a very similar slope to that obtained in Experiment Two with an x-axis intercept at  $0.22 \text{ g Chl m}^{-2}$  (Fig 5.17 c).

The dashed lines in panels (a) and (c) of Figure 5.17, represent the relationship of  $0.42 \text{ g CF}_1 \text{ g}^{-1} \text{ Chl}$  obtained for spinach by Strotmann et al., (1973). However, the line has been plotted assuming a zero intercept, as one is unable to estimate the actual intercept from the data presented in this paper. Nevertheless, the slopes obtained in both experiments of this study, show close agreement with that obtained by Strotmann et al., (1973).

#### 5.4.5 Discussion

For the relationships of Chlorophyll, Chl a/b and soluble protein versus leaf N content (Figs 5.12, 5.13 and 5.14 respectively), good reproducibility has been demonstrated, regardless of whether these relationships have been derived from leaf samples principally extracted to determine Rubisco content (as in Chapter Four, Figures 4.7, 4.8 and 4.9), or as here, extracted to determine  $\text{CF}_1$  content. This demonstration is important, firstly because it shows that the methods used to determine chlorophyll and soluble protein are consistent and reliable. Secondly, it demonstrates that there is minimal variation between leaf halves from which either Rubisco or  $\text{CF}_1$  has been extracted on different dates (as in Experiment Two), or that variation within replicate leaves for a given treatment and occasion, from half of which Rubisco was extracted and the other half  $\text{CF}_1$ , is also small (as applicable to Experiment Three). Further, in both this and the preceding chapter, the relationship between Chlorophyll and leaf N content has been shown to be comparable to the same relationship obtained by Evans (1983) for wheat, and for soluble protein versus

N, comparable to the relationship obtained by Evans and Austin (1986), also for wheat.

In view of the evidence presented in Chapter Four, that long-term growth at 70 Pa CO<sub>2</sub> had no significant effect in altering the amount of N invested in Rubisco as a proportion of leaf N, the specific hypothesis being tested in this chapter is that, if an inherent mechanism does exist and operated in the plants of this study to redress the balance of capacities at 70 Pa CO<sub>2</sub>, then we would expect to see an increase in CF<sub>1</sub> content as a proportion of leaf N. In the preceding discussion to this (5.4.3), it was suggested that on a leaf area basis, whilst both the amount of CF<sub>1</sub> and N decreased faster in leaves grown at 70 Pa CO<sub>2</sub> compared to those at 36 Pa CO<sub>2</sub>, leaf N content may still have decreased proportionately faster than CF<sub>1</sub> in 70 Pa CO<sub>2</sub> grown leaves, particularly in the low N treatment. This meant that the possibility of some re-balancing between capacities could not be ruled out. However, that this was not the case, is confirmed by the data presented in Figure 5.15, in which the relationship between CF<sub>1</sub> and leaf N content is demonstrated to be independent of CO<sub>2</sub> and N treatment. Whilst the actual slopes and intercepts for this relationship in leaves of Experiment Two and Three (Fig. 5.15 a & c respectively) are not directly comparable, the observation that there was no direct CO<sub>2</sub> effect, is consistent. Therefore, there was no evidence in this study to suggest that CF<sub>1</sub> content as a proportion of leaf N was affected by long-term growth at 70 Pa CO<sub>2</sub>.

As reported in the introduction to this chapter, no references in the literature for which the relationship between CF<sub>1</sub> and leaf N content has been thoroughly investigated in wheat have been found, and certainly not with varying CO<sub>2</sub> treatments. However, in Makino et al., (1997a; 1997b), data are presented for the relationship

between  $CF_1$  and leaf N content in leaves of rice grown at different irradiance, or analysed from wild-type plants, but not subjected to  $CO_2$  treatment. The relationship between these experiments for control or wild-type plants was linear as found for this study.

In an experiment in which rice was grown hydroponically at either 36 or 70 Pa  $CO_2$ , Nakano et al., (1997) reported no significant effect of  $CO_2$  on the relationship between Cytochrome *f* and total leaf N content in leaves measured at full emergence. Since Cytochrome *f* like  $CF_1$  is also a major determinant of RuBP-regeneration capacity, the finding of Nakano et al., (1997) may be taken as indirect support for the conclusion in this study relating to  $CF_1$ .

In section 5.4.3, it was suggested that the values for  $CF_1$  content in high N leaves of the third and fourth occasion in Experiment Three could have been substantially underestimated. The relationships for  $CF_1$  versus N and  $CF_1$  versus Chl obtained when all data points for Experiment Three were plotted (Figs 5.15 b and 5.17 b respectively), appear to support this argument, since the  $R^2$  values for these relationships, the degree of comparison to results for these relationships in Experiment Two, and comparison with the  $CF_1$ :Chl relationship found by Strotmann et al., (1973), all improved dramatically (Figs 5.15 c and 5.17 c) when the data points in question were removed. Further, since as suggested above, we can be confident about estimations of leaf Chl and N content, the only estimation which can possibly be erroneous is that for  $CF_1$ .

## 5.5 Conclusions

From results obtained over the course of two separate experiments, in which leaf samples were taken from full emergence through to senescence, the long-term growth of *T. aestivum* L. cv Minaret at 70 compared to 36 Pa CO<sub>2</sub>, had no significant effect on amounts of CF<sub>1</sub> in leaves on an area basis, in any treatment of any occasion. Prior to at least six days after anthesis, the same was also generally true for leaf N content, but thereafter some significant effects of the CO<sub>2</sub> treatments were observed, with amounts of N being lower in the 70 Pa CO<sub>2</sub> grown plants. However, there was no evidence for a slower decline in the amount of CF<sub>1</sub> as a proportion of total leaf N on any occasion (the hypothesis being specifically tested here in view of evidence from Chapter Four). There was therefore no evidence of an ideal biochemical acclimation to elevated CO<sub>2</sub> in these plants. Similarly, in Chapter Four, no evidence was found for a preferential decline in the amount of Rubisco as a proportion of total leaf N content on any occasion. Thus when both of these conclusions are taken together, we can conclude that there is no evidence in this study to suggest that a reallocation of N away from the main component of the least limiting capacity of carboxylation (Rubisco), to one of the main components of the limiting capacity of RuBP-regeneration (CF<sub>1</sub>), took place in plants grown in this study at 70 Pa CO<sub>2</sub> and either low or high N applications. This conclusion is despite a 17% decrease in the initial activity of Rubisco in plants shortly after full leaf expansion (grown and sampled at 100 Pa CO<sub>2</sub> and saturating light; Chapter Four), suggesting that carboxylation capacity was excessive of requirements, so that some reallocation of resources could have taken place to increase efficiency.

In agreement with results from the previous chapter on Rubisco, long-term growth at 70 Pa CO<sub>2</sub> for leaves principally extracted to determine CF<sub>1</sub> content, had no



significant effect on the relationships of Chl or soluble protein as a function of leaf N. Given this fact, and the early but parallel loss of CF<sub>1</sub> and N in 70 Pa CO<sub>2</sub> grown leaves, this suggests that the only effect of growing leaves at 70 Pa CO<sub>2</sub> was to bring forward the onset of senescence.

This overall conclusion, is contrary to that found in the experiment of Nie et al., (1995a), in which spring wheat was grown at either 36 or 55 Pa CO<sub>2</sub> under FACE, and for which it was concluded that in flag leaves subsequent to anthesis, a CO<sub>2</sub> specific decline in the amount of Rubisco relative to other components, including CF<sub>1</sub>, occurred. However, it has already been demonstrated previously in Chapter Four, that the proportionalities between Rubisco, soluble protein and Chl content can change merely as a function of total leaf N, and likewise the same has been demonstrated in this chapter for CF<sub>1</sub>, soluble protein and Chl. This begs the question, do CF<sub>1</sub> and Rubisco decline in parallel as a function of leaf N content? If they do not, then this could explain the observation of Nie et al., (1995a). Thus, one of the aims of the next and final chapter, is to address this question, as well as bringing together data and results from other chapters and make final conclusions.



## CHAPTER 6

The effect of elevated partial pressures of CO<sub>2</sub> on the relationship between Rubisco and the thylakoid ATP-synthase, their relationships with parameters of photosynthesis, and estimation of the excess investment in Rubisco

### 6.1 Introduction

In this final chapter, data from previous chapters are brought together and re-analysed to examine in more detail the effect of elevated  $pC_a$  on the balance of RuBP-regeneration and carboxylation capacities. While the findings in previous chapters have already shown that the large shift in this balance, predicted as optimal by theory (Medlyn, 1997), does not occur, here the extent of any shift is quantified, evidence is established as to whether this is directly attributable to elevated  $pC_a$ , and the consequences for N-use in leaves are estimated.

The first aim of this chapter is to determine the relationship between the amounts of Rubisco and thylakoid ATP-synthase in leaves of different treatments, to search for any evidence of a lower Rubisco:thylakoid ATP-synthase ratio at elevated  $pC_a$ . This will be done by looking at this relationship as a function of absolute amount per unit leaf area as there is evidence from previous chapters that this may explain apparent effects of  $pC_a$ , e.g. the decreased Rubisco:CF<sub>1</sub> ratio found at elevated  $pC_a$  by Nie et al., (1995a), in wheat grown under FACE.

A second aim is to investigate the relationships between Rubisco, CF<sub>1</sub> and photosynthetic parameters derived from the gas exchange data previously reported in Chapter Three, and relate results to those available elsewhere in the literature. However, since the limited data available in the literature predominantly concerns the whole thylakoid ATP-synthase, conversion of the CF<sub>1</sub> data reported in Chapter Five is

necessary. This has been done assuming that all CF<sub>1</sub>  $\alpha$ - and  $\beta$ -subunits are present in the thylakoid ATP-synthase complex, and then multiplying CF<sub>1</sub>  $\alpha$  and  $\beta$  content by 1.61 g ATP-synthase per g CF<sub>1</sub>  $\alpha$  and  $\beta$  (Moase and Green, 1981).

A third objective, given that there is little evidence in this study for biochemical acclimation within the photosynthetic machinery, despite decreased initial Rubisco activity indicating that carboxylation capacity was excessive of requirements, is to estimate the amount of Rubisco that was in excess of the requirement for leaf photosynthesis in plants grown at elevated  $pC_a$ . If a sufficient amount of excess can be demonstrated, such results may justify attempts to increase the N-use efficiency of photosynthesis by genetic manipulation for this species or others, for higher CO<sub>2</sub> environments of the future. Finally, at the end of the chapter, conclusions for this study will be presented.

## **6.2 The relationship between Rubisco and the thylakoid ATP-synthase**

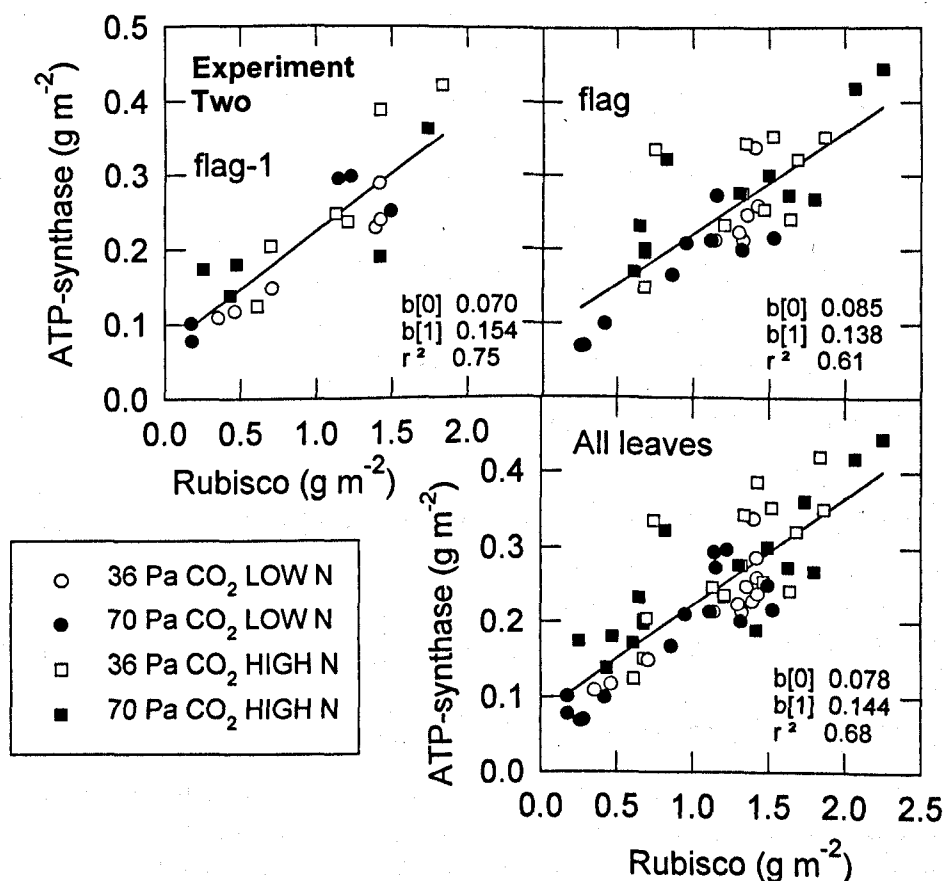
### **6.2.1 Introduction**

To determine whether the mass ratio between Rubisco and the thylakoid ATP-synthase is constant or changes as leaves age and lose N, the relationship between these two components both measured directly in the same leaf sample and only available for Experiment Two, were determined for flag-1 and flag leaves of various ages.

### **6.2.2 Results**

Figure 6.1 below clearly shows for each leaf insertion, that Rubisco and ATP-synthase are not simply proportional, and therefore that the mass ratio of Rubisco to

ATP-synthase is not constant. For the combined data (bottom panel) the mass ratio of Rubisco to ATP-synthase increases from about 3.8 at low Rubisco content up to 5.75 at high Rubisco content. Therefore, as leaf N is remobilised from leaves Rubisco will be entirely removed before the thylakoid ATP-synthase. Importantly, there are no significant effects of N or CO<sub>2</sub> treatment on this relationship.



**Figure 6.1** The relationship between ATP-synthase and Rubisco contents measured directly in the same leaf sample for flag-1 and flag leaves of Experiment Two. Data for both leaves are combined in the bottom panel. Lines are first order linear regressions. Plants were grown under regimes as denoted by the key.

### 6.2.3 Discussion

The relationship between ATP-synthase and Rubisco content determined in Experiment Two (Fig. 6.1), was such that the ratio of Rubisco to ATP-synthase increased with increasing Rubisco content, independent of treatment. Since amounts of these components are expected to reflect carboxylation and RuBP-regeneration

capacities (supported by the data below in Figs. 6.2 and 6.3), this suggests that carboxylation capacity increases relative to RuBP-regeneration capacity in leaves with high N content, and that this is not affected by elevated CO<sub>2</sub>. This result is in close agreement with that of Nakano et al. (1997) who grew rice at elevated  $pC_a$ . The findings of Nie et al., (1995a) in a field experiment of wheat under FACE, where elevated CO<sub>2</sub> did not affect the ratio of Rubisco to other components, including CF<sub>1</sub> at flag leaf emergence, but did induce a decrease in this ratio during grain-fill, could therefore be interpreted simply in terms of a more rapid decline in leaf N in the elevated CO<sub>2</sub>-grown leaves, as opposed to a direct CO<sub>2</sub> effect on Rubisco as the authors suggest.

### **6.3 Relationships between Rubisco, the thylakoid ATP-synthase, and photosynthetic parameters**

#### **6.3.1 Introduction**

In Chapter Three,  $A/pC_i$  data were presented with the minimum of interpretation. Whilst this is a commonly adopted approach, it nevertheless takes no account of variation due to dark respiration or changes in the conductance for diffusion of CO<sub>2</sub> from the intercellular spaces of leaves to the sites of carboxylation ( $g_w$ ). Therefore to try and account for these factors as is frequently done (Besford et al., 1990; van Oosten et al., 1995), the gas exchange data obtained in experiments Two to Four, has been re-analysed using the following procedure.

### 6.3.2 Method

Light-saturated photosynthesis was assumed to be determined by the RuBP-saturated kinetics of Rubisco for  $pC_i < 35$  Pa (Makino et al., 1988), and points in this region were fitted to equation 6.1 below. In about 10% of cases, this procedure gave a poor fit ( $R^2 < 0.90$ ), in which case the fit was restricted to points with  $pC_i < 25$  Pa, which invariably increased  $R^2$  to above 0.90. The responses were fitted assuming a finite transfer conductance to diffusion of  $CO_2$  from the intercellular space to the site of carboxylation ( $g_w$ ) (Loreto et al., 1992), so that photosynthetic rate gross of non-photorespiratory respiration ( $A_g$ ), is given by (see von Caemmerer and Evans, 1991):

$$A_g = \frac{1}{2}(K \cdot g_w + p_i \cdot g_w + R_d + V_{cmax} - \Delta),$$

$$\Delta = \sqrt{K^2 \cdot g_w^2 + 2 \cdot K \cdot g_w^2 \cdot p_i + 2 \cdot K \cdot g_w \cdot R_d + 2 \cdot K \cdot g_w \cdot V_{cmax} + p_i^2 \cdot g_w^2 + 2 \cdot p_i \cdot g_w \cdot R_d - 2 \cdot V_{cmax} \cdot p_i \cdot g_w + R_d^2 - 2 \cdot V_{cmax} \cdot R_d + V_{cmax}^2 + 4 \cdot V_{cmax} \cdot \Gamma^* \cdot g_w}$$

[6.1]

where  $K$  is the effective Michaelis-Menten constant for  $CO_2$  at 20 kPa  $O_2$ ,  $\Gamma^*$  is the photorespiratory compensation point,  $V_{cmax}$  is the maximal carboxylation rate and  $R_d$  is the rate of non-photorespiratory respiration. The values of  $K$  and  $\Gamma^*$  at 20°C were assumed constant for all leaves and were taken from the literature:  $K=47.0$  Pa (value for wheat at 25°C taken from Makino et al. (1988); temperature dependence from Machler et al. (1980) and Keys (1999),  $\Gamma^* = 3.4$  Pa (Brooks and Farquhar, 1985). As the value of  $g_w$  cannot practically be estimated by fitting  $A/pC_i$  responses (von Caemmerer et al., 1994), three different approaches were tried for this parameter for all response curves. In the first,  $g_w$  was assumed to have a constant value for all leaves. In the second, it varied between leaves such that the  $CO_2$  partial pressure at the site of carboxylation was 0.55 of atmospheric ambient, i.e. 20 Pa

when  $pC_a=36$  Pa (Farquhar and Sharkey, 1994). In the third, an initial value of  $5.0 \mu\text{mol m}^{-2} \text{s}^{-1} \text{Pa}^{-1}$  at leaf emergence, declining as the leaf senesced to a value of  $2.0 \mu\text{mol m}^{-2} \text{s}^{-1} \text{Pa}^{-1}$ , was used based on the dependence on leaf age found for wheat flag leaves (Loreto et al., 1994). Since the last approach gave the best  $R^2$  values ( $>0.95$  for 96% and  $>0.99$  for 74% of curves), all the data presented from the 508 response curves in the three experiments were obtained using this approach. (However, as  $g_w$  was not measured, the sensitivity of our conclusions to the assumed value of  $g_w$  is addressed at appropriate points in the text). Thus for each  $A/pC_i$  response curve, non-linear regression (Genstat5, Rothamsted Experimental Station) was used to estimate the values of only two parameters in Equation 6.1;  $V_{\text{cmax}}$  and  $R_d$ .

For analysing the relationships between components and photosynthetic parameters, the  $k_{\text{cat}}$  for Rubisco was estimated by the coefficient relating  $V_{\text{cmax}}$  to the Rubisco content, estimated by linear regression forced through the origin, and converted to units  $\text{mol CO}_2 (\text{mol active site})^{-1} \text{s}^{-1}$ .

The  $k_{\text{cat}}$  for thylakoid ATP-synthase can be estimated from the relationship between  $A_{g,70}$  and ATP-synthase content. The rate of ATP synthesis ( $V_{\text{ATP}}$ ) required to maintain a photosynthetic rate at  $pC_a=70$  Pa is given by (Farquhar and Caemmerer, (1982) Eq. 16.27):

$$V_{\text{ATP}} = \frac{3 \cdot p_c + 7 \cdot \Gamma_*}{p_c - \Gamma_*} \cdot A_{g,70} \quad [6.2]$$

where  $p_c$  is the  $\text{CO}_2$  partial pressure at the site of carboxylation, given by  $p_i A/g_w$ . Since estimates of  $p_c$  did not vary much,  $V_{\text{ATP}}$  was well approximated by  $3.68 A_{g,70}$ .

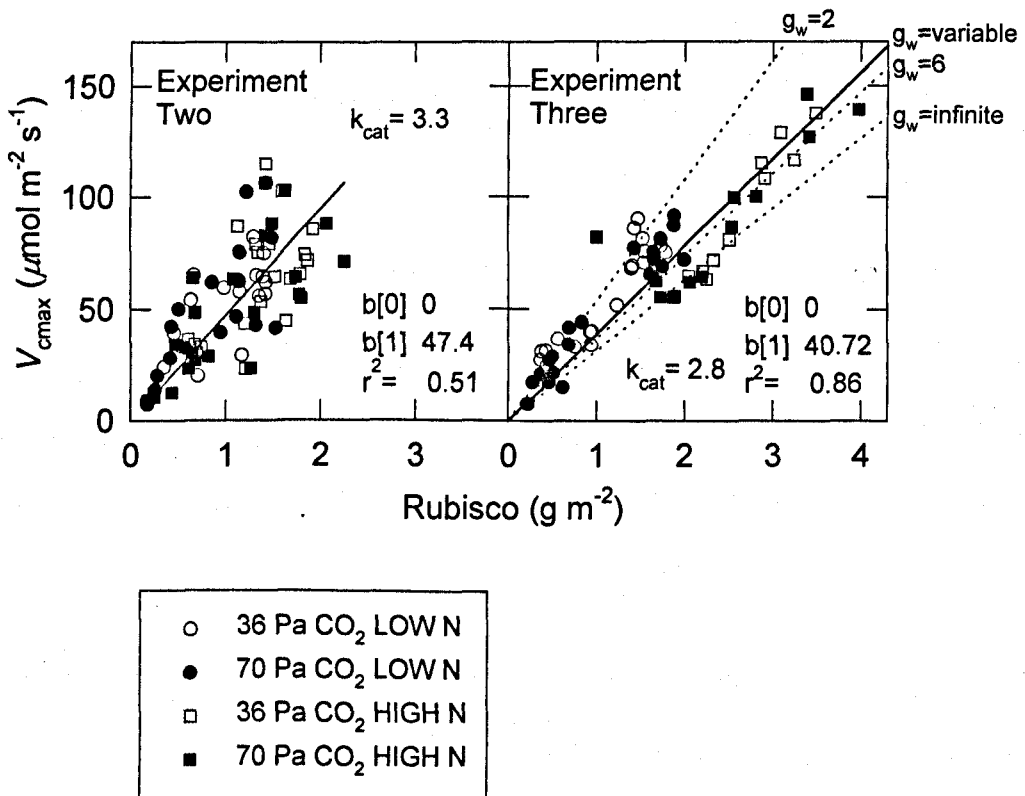


Linear regression forced through the origin of  $A_{g,70}$  on the amount of thylakoid ATP-synthase gives a coefficient which is therefore approximately proportional to  $k_{cat}$ . This is multiplied by the 3.68 factor and by  $0.555 \text{ g } \mu\text{mol}^{-1}$  to convert to units of  $\text{mol ATP (mol ATP-synthase)}^{-1} \text{ s}^{-1}$ .

### 6.3.3 Results

The responses through time of carboxylation capacity,  $V_{cmax}$ , as estimated from  $A/pC_i$  responses, and photosynthetic rates at 70 Pa  $\text{CO}_2$  external partial pressure and high light, corrected for non-photorespiratory respiration, ( $A_{g,70}$ ), gave very similar patterns of response to those obtained previously for  $\epsilon$  and  $A_{max}$  (Figs. 3.4 and 3.5 respectively) in Chapter Three (i.e. no significant effect of growth  $\text{CO}_2$  conditions on either parameter at full leaf expansion or prior to anthesis, but with any subsequent effect causing a more rapid decline), and so are not shown here but have previously been presented in Theobald et al., (1998).

Figure 6.2 shows the relationship between  $V_{cmax}$  estimated from  $A/pC_i$  responses, and Rubisco content of the same leaf samples, for the combined data of flag-1 and flag leaves in Experiment Two, and for flag leaves in Experiment Three, for which growth  $\text{CO}_2$  partial pressure or N treatment had no significant effect. For both experiments, the relationships were close to theoretical proportionality, the linear regression through the origin for Experiment two ( $r^2 = 0.51$ ) giving an overall estimate for  $k_{cat}$  of 3.3, and the regression (solid line) of Experiment Three ( $r^2 = 0.86$ ) giving an estimated  $k_{cat}$  of 2.8 (SE  $0.06 \text{ s}^{-1}$ ).

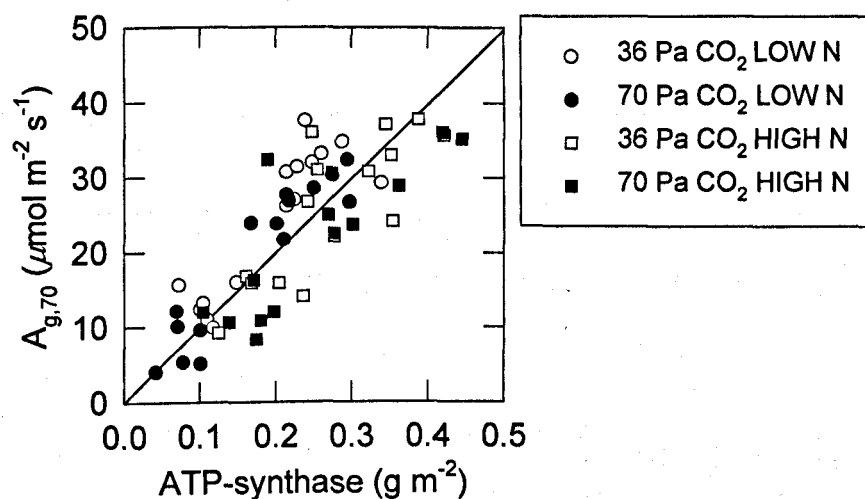


**Figure 6.2** Rate of estimated (see text) maximum carboxylation ( $V_{cmax}$ ), versus Rubisco content, for flag-1 and flag leaves of Experiment Two, and the first four occasions of flag leaves from Experiment Three. Symbols and treatments are indicated by the key. Lines are linear regressions forced through the origin. For Experiment Three, dotted lines are equivalent regressions for the same data when recalculated with  $g_w = 2$ , 6 or infinite  $\mu\text{mol m}^{-2} \text{s}^{-1} \text{Pa}^{-1}$  ( $r^2 = 0.75, 0.67$  and  $0.56$  respectively). For further details see text.

However, as  $g_w$  was not measured but was assumed to be variable, the sensitivity of estimates for  $V_{cmax}$  and in turn  $k_{cat}$  were tested for the data of Experiment Three, by assuming constant  $g_w$  values of 2.0, 6.0  $\mu\text{mol m}^{-2} \text{s}^{-1} \text{Pa}^{-1}$  (spanning the range estimated in measurements: von Caemmerer and Evans, 1991; Loreto et al., 1994) or infinity. For the regression between  $V_{cmax}$  and Rubisco content, these gave  $k_{cat}$  estimates of 3.71, 2.53, 2.17  $\text{s}^{-1}$  with  $R^2 = 0.75, 0.67, 0.56$ , respectively, compared to 2.79  $\text{s}^{-1}$ ;  $R^2 = 0.83$  for data in Figure 6.2.

The relationship between gross photosynthetic rate measured at 70 Pa  $\text{CO}_2$  partial pressure and ATP-synthase content of the same leaf samples is shown in

Figure 6.3, for flag-1 and flag leaves of Experiment Two. The line shown is a linear regression through the origin ( $R^2=0.65$ ) and from the slope a  $k_{\text{cat}}$  was estimated using Equation 6.2 of  $200 \text{ mol ATP (mol ATP-synthase)}^{-1} \text{ s}^{-1}$  (SE 6).



**Figure 6.3** Rate of estimated photosynthesis gross of dark respiration at 70 Pa  $\text{CO}_2$  ( $A_{g70}$ ), versus ATP-synthase content, for flag-1 and flag leaves of Experiment Two. Photosynthesis was measured at a leaf temperature of  $20^\circ\text{C}$ , a PPFD of  $1500 \mu\text{mol PPFD m}^{-2} \text{ s}^{-1}$  and a leaf-to-air vapour pressure deficit of 1.5 kPa. Plants were grown under the regimes indicated by the key. The line is a linear regression forced through the origin,  $Y=98 X$ ,  $R^2=0.65$ .

#### 6.3.4 Discussion

The relationship between the estimated *in vivo*  $V_{\text{cmax}}$  and Rubisco content used to derive the estimates of Rubisco  $k_{\text{cat}}$  of 3.3 and  $2.8 \text{ s}^{-1}$  at  $20^\circ\text{C}$  (Fig. 6.2) for Experiments Two and Three, was strongest for the latter ( $R^2=0.83$ ). These results were further supported by the assumption of a variable  $g_w$  compared to fixed values (tested for Experiment Three) of 2, 6 or infinite, which improved the fit between Rubisco content and photosynthetic parameters, as found previously for wheat (Evans and Austin, 1986; Makino et al., 1988). Indeed, these values are in line with *in vivo* estimates for tobacco of  $3.5 \text{ s}^{-1}$  ( $25^\circ\text{C}$ ) (von Caemmerer et al., 1994), and for wheat of  $3.3 \text{ s}^{-1}$  ( $23^\circ\text{C}$ ) (Evans and Austin, 1986) and an *in vitro* estimate for wheat of  $3.0 \text{ s}^{-1}$  ( $25^\circ\text{C}$ ) (Makino et al., 1988), given the temperature dependence which

would be expected to increase the value by 40% going from 20 to 25°C (Machler et al., 1980; Makino et al., 1988).

Gross photosynthetic rate at 70 Pa CO<sub>2</sub> and ATP-synthase content were well correlated (Fig. 6.3), and resulted in a  $k_{\text{cat}}$  estimate for ATP-synthase (200 s<sup>-1</sup>) which is comparable to that obtained *in vitro* (160 s<sup>-1</sup>; Fromme and Graber, 1989). A strong relationship is expected because nearly all products of photosynthetic electron transport are used in RuBP-regeneration for photosynthesis and photorespiration (Habash et al., 1995) and the photosynthetic rate is usually limited by RuBP-regeneration at the  $pC_i$  experienced here (45-55 Pa) (Makino et al., 1988). Although under these conditions rate can also be limited by triose-phosphate utilisation capacity (Farquhar and Sharkey, 1994), in our experiments the stimulation of  $A_{g,70}$  on decreasing O<sub>2</sub> partial pressure to 2 kPa were mostly close to the theoretical expectation for RuBP-regeneration limited photosynthesis (mean stimulation 18%; Chapter Three).

These observations for the relationships between  $V_{\text{cmax}}$  and Rubisco and the thylakoid ATP-synthase and  $A_{g,70}$ , support the expectation that the amount of these components determine carboxylation and RuBP-regeneration capacities respectively, and as such,  $V_{\text{cmax}}$  in particular can be used as a reliable estimate of the amount of Rubisco.

## 6.4 Estimation of excess Rubisco in leaves grown at 70 Pa CO<sub>2</sub>

### 6.4.1 Method

For any particular observed value of  $A$  at a given  $pC_i$ , the minimum value of  $V_{\text{cmax}}$  required to achieve this ( $V_{\text{cmax,req}}$ ) from Eq. 1 is:

$$V_{c\max,req} = A_g \cdot \frac{p_i \cdot g_w + K \cdot g_w - A}{p_i \cdot g_w - \Gamma \cdot g_w - A} \quad [6.3]$$

The amount of apparent excess investment in Rubisco ( $R_{xs}$ ) compared to that needed to maintain the photosynthetic rate is given by:

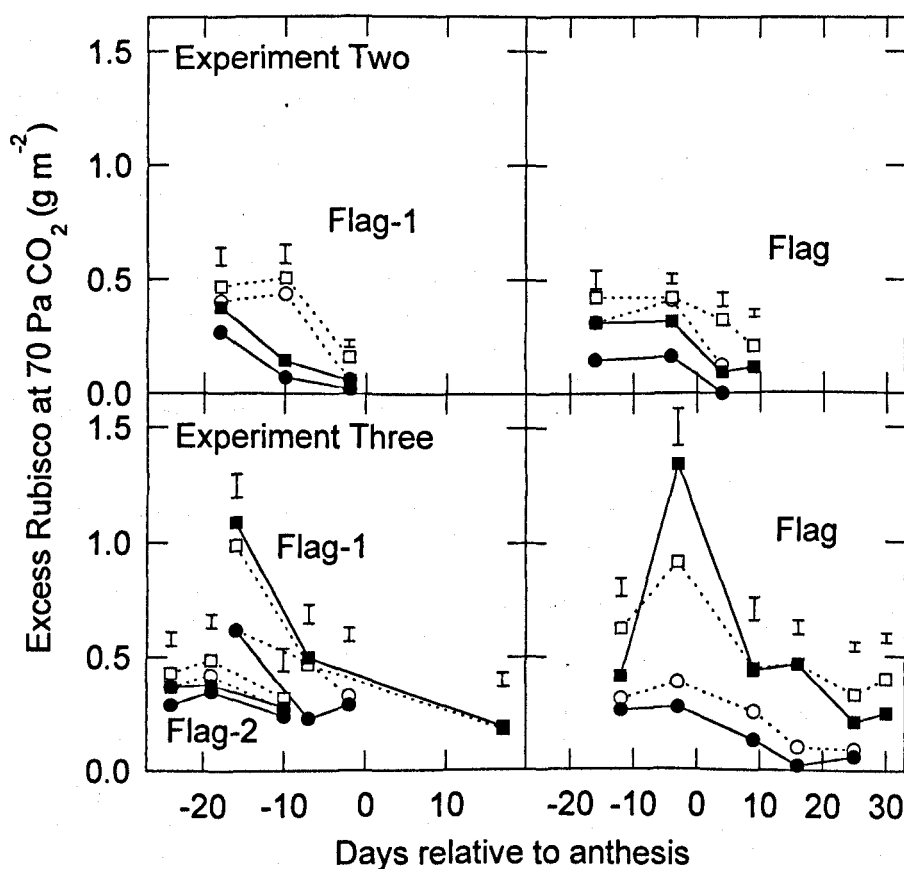
$$R_{xs} = \frac{V_{c\max} - V_{c\max,req}}{8 \cdot k_{cat}} \cdot 0.55 \quad [6.4]$$

where  $k_{cat}$  is maximal rate of carboxylation per active site on fully-activated Rubisco and 8 is the number of active sites per molecule and 0.55 the molecular weight (g  $\mu\text{mol}^{-1}$ ) for Rubisco. Equations 6.2 and 6.3 were used to estimate the theoretical excess in Rubisco content compared to that required to maintain the observed assimilation rate with the external  $\text{CO}_2$  partial pressure set at the elevated  $\text{CO}_2$  growth value. Thus Equation 6.3 was used with the observed  $pC_i$  and  $A$  values corresponding to  $pC_a=70$  Pa for Experiments Two and Three, and  $pC_a=100$  Pa for Experiment Four. In only 4% of the  $A/pC_i$  responses were these estimated values of  $R_{xs}$  less than zero, and these were within the error of the fitting procedure, suggesting that the assumed values of  $K$ ,  $\Gamma$  and variable  $g_w$ , as supported by the analysis for estimation of  $V_{c\max}$  (Fig. 6.2), were reasonable.

#### 6.4.2 Results

Given the relationship between  $V_{c\max}$  and Rubisco content (Fig. 6.2), Equation 6.4 was used to estimate the apparent excess Rubisco content compared to that required to maintain the observed rate of photosynthesis at 70 Pa atmospheric  $\text{CO}_2$  (Fig. 6.4). For all leaves, and for both experiments, the excess is consistently less at low N. Where elevated  $\text{CO}_2$  treatment has a significant effect (in Experiment Two flag-1 leaves, and for flag leaves in all Experiments), it decreases the excess. In

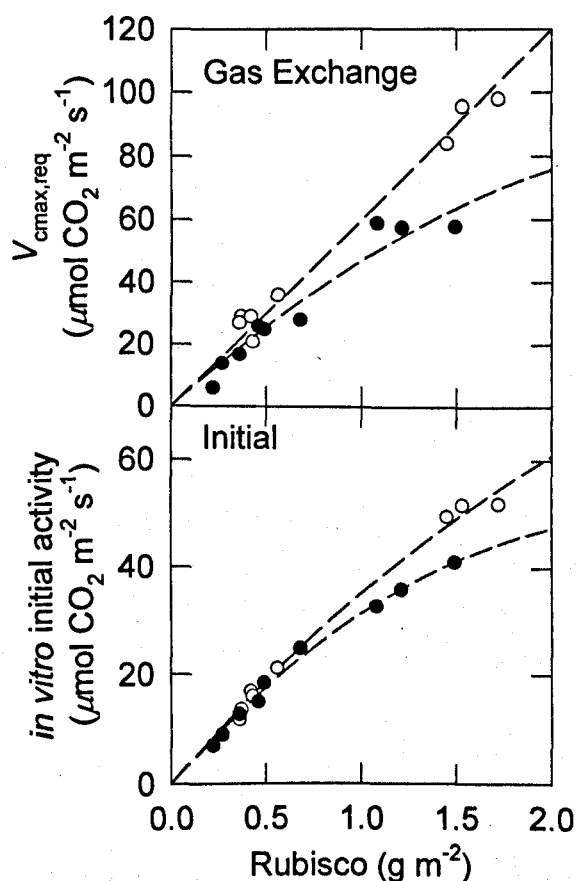
some cases (e.g. Experiment Three, low N 70 Pa flag leaves after 15 days from anthesis), the estimated excess was not significantly greater than zero. It is also possible to use Equation 6.4 to estimate excess Rubisco at ambient  $\text{CO}_2$ . This was only significantly different from zero for the last three points for high N flag leaves in Experiment Three, decreasing from  $\sim 0.4$  to  $\sim 0.2 \text{ g m}^{-2}$  (data not shown).



**Figure 6.4** The estimated excess (see text) of Rubisco at 70 Pa  $\text{CO}_2$  atmospheric partial pressure versus time as days relative to anthesis, for various wheat leaves from two experiments. Error bars represent SE of differences between mean values shown from  $\text{CO}_2 \times \text{N}$  analysis of variance for each occasion ( $n=6$ ). Symbols and treatments are as indicated in the key of Figure 6.3.

In Experiment Four, leaves used for gas exchange were freeze-clamped at high light and at their growth  $pC_a$ , and the activity of isolated Rubisco was determined *in vitro* and related to the Rubisco content, as has been discussed previously in Chapter Four (section 4.6, Fig. 4.13). In Figure 6.5, the *in vitro* initial activities taken from Figure 4.13 are compared to the  $V_{\text{cmax}}$  required to achieve the

observed photosynthetic rate measured at the growth  $\text{CO}_2$  partial pressure, estimated from the gas exchange parameters using Equation 6.3.



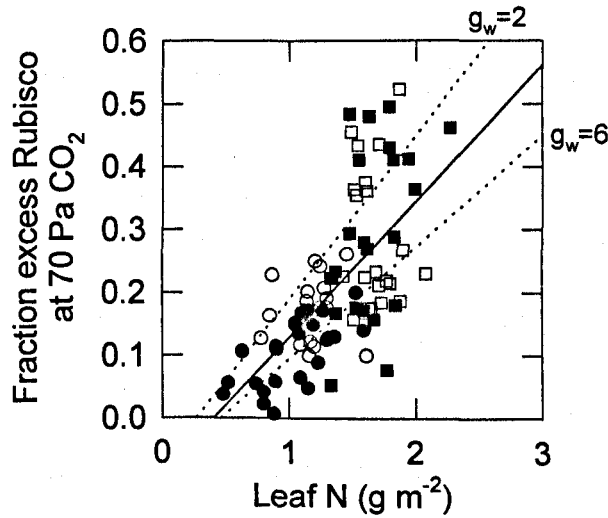
**Figure 6.5** Estimates of *in vivo*  $V_{\text{cmax}}$  required (top panel) to maintain observed photosynthetic rate at a PPFD of  $1500 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$  and the growth  $\text{CO}_2$  partial pressure (estimated from the  $A/pC_i$  response using Eq. 2) versus Rubisco content in flag leaves of different ages measured between 22 d before anthesis until 18 d after anthesis in Experiment Four. Plants were grown at 36 (open symbols) and 100 (closed symbols) Pa  $\text{CO}_2$  partial pressure. Lines are first order linear regressions through the origin, or second order where this improved  $r^2$ . The bottom panel has previously been shown in Fig 4.14, but is reshown for ease of comparison.

The initial activities show a distinct curvilinear relationship with the amount of Rubisco determined by  $[^{14}\text{C}]$ -CABP binding, and the lines for the two  $\text{CO}_2$  treatments diverge at high Rubisco content. A similar pattern is seen for the estimates of required  $V_{\text{cmax}}$ , which diverge at higher Rubisco contents.

### 6.4.3 Discussion

The amount of Rubisco in excess of the requirement to maintain photosynthesis at high light and  $pC_a=70$  Pa, estimated from the gas exchange data, (Fig. 6.4) showed a clear pattern in all leaves, where the excess was greater in high N compared to low N leaves, and declined as leaves senesced. The effect of elevated  $CO_2$  treatment was more variable, but generally decreased the excess. It is therefore clear that some re-balancing of capacities does occur in the elevated  $CO_2$  treatment, since the excess is lower, but there is still some excess until well into senescence in the low N treatments, and throughout in the high N treatments. From the above and the conclusions of Nakano et al. (1997), such rebalancing can be explained purely in terms of elevated  $CO_2$  treatment inducing a decrease in leaf N. This is associated with a greater than proportional decrease in Rubisco (Fig. 4.10 in Chapter Four), compared to all other photosynthetic components, in particular ATP-synthase (Fig. 6.1), so that there is a *de facto* rebalancing away from carboxylation to RuBP-regeneration capacities. This hypothesis is supported by the relationship between the data in Fig. 6.4, expressed as the fraction of Rubisco in excess at 70 Pa  $CO_2$  versus leaf N (Fig. 6.6). The fraction increases from ~5% in leaves with 1 g N m<sup>-2</sup> to ~40% in leaves with 2 g N m<sup>-2</sup>, regardless of  $CO_2$  treatment. Thus any decrease in excess Rubisco due to elevated growth  $CO_2$  partial pressure appears to be an indirect consequence of decreased leaf N.





**Figure 6.6** The fraction of Rubisco estimated to be in excess of that required to maintain photosynthetic rate at 70 Pa atmospheric  $\text{CO}_2$  partial pressure versus total leaf N content, for all leaves in Experiments Two and Three. Plants were grown at low (circles) and high (squares) N applications, and at 36 (open symbols) or 70 (closed symbols) Pa  $\text{CO}_2$ . Solid line is linear regression of all points;  $R^2=0.41$ ,  $n=87$ . Dotted lines are equivalent regressions for same data when recalculated with  $g_w=2$  and  $6 \mu\text{mol m}^{-2} \text{s}^{-1} \text{Pa}^{-1}$ ;  $R^2=0.56$ ,  $0.45$ , respectively.

The estimates of excess Rubisco refer to the conditions used for determination of steady-state photosynthesis. The conditions of high light intensity and moderate temperature used are those under which Rubisco would be expected to be least in excess, so that the excess would be, if anything, greater under variable field conditions at elevated  $\text{CO}_2$ . (However, this conclusion would not hold for conditions which decrease stomatal conductance, such as water stress.)

However, as for the estimations of  $V_{\text{cmax}}$  and  $k_{\text{cat}}$ , the analysis above for estimating excess Rubisco assumes a variable  $g_w$ . Therefore, in order to investigate the sensitivity of estimations of excess Rubisco to this assumption, the analysis was repeated with the same constant  $g_w$  values of  $2.0$ ,  $6.0 \mu\text{mol m}^{-2} \text{s}^{-1} \text{Pa}^{-1}$  or infinity. In contrast to the marked effect of  $g_w$  on  $k_{\text{cat}}$ , the value of  $g_w$  had much less effect on the main aim of estimating the fraction of Rubisco that is in excess at elevated  $\text{CO}_2$ . The dotted lines in Fig. 6.6 show the corresponding linear regressions with  $g_w=2$

and  $6 \mu\text{mol m}^{-2} \text{s}^{-1} \text{Pa}^{-1}$ . These values would alter the estimate of 40% excess Rubisco at  $2 \text{ g N m}^{-2}$  to 50% and 30%, respectively. Even making the unlikely assumption of an infinite  $g_w$  gave the same highly significant trend and a corresponding fraction excess of 20% (data not shown).

### *Rubisco activation*

It is usually assumed that Rubisco in excess of that required to maintain carboxylation will be deactivated, since RuBP concentrations are stable (Sage et al., 1990) and a decrease in Rubisco activation state at elevated  $\text{CO}_2$  has been found in some studies (Sage et al., 1990; Nakano et al., 1997), but not all (e.g. Sicher et al., 1995). The estimated value of the  $V_{\text{cmax}}$  required to maintain observed photosynthetic rate ( $V_{\text{cmax,req}}$  from Eq. 6.3) should therefore be directly comparable to the initial activity of Rubisco. Activity parameters determined for Rubisco isolated from leaf sections freeze clamped under high light and growth  $\text{CO}_2$  partial pressure, were compared with  $V_{\text{cmax,req}}$  values estimated for the same leaf sections under these conditions (Fig. 6.5). The pattern of  $V_{\text{cmax,req}}$  is similar to initial activity, with elevated  $\text{CO}_2$  points lying increasingly below ambient  $\text{CO}_2$  points as Rubisco content increases, although the absolute initial *in vitro* activities are much lower. Estimates of Rubisco activity are often higher *in vivo* than *in vitro* (von Caemmerer et al., 1994), and other possible explanations have previously been discussed in Chapter Four. Despite this, the differences between  $\text{CO}_2$  treatments probably do reflect the *in vivo* state.

## 6.5 Conclusions

In this chapter we have found evidence, (1) from the similarities between the

relationships for estimated  $V_{\text{cmax,req}}$  and the measured initial activity of Rubisco verses the amount of enzyme (Fig.6.5), and (2) from estimates of excess Rubisco (Figs 6.4 and 6.6), to suggest that there is excess investment in Rubisco over that required for maintaining photosynthetic rate at elevated  $\text{CO}_2$  and that this excess is greater in leaves with high N content. Long term growth at elevated  $\text{CO}_2$  does decrease this excess somewhat, but apparently only as an indirect consequence of elevated  $\text{CO}_2$  causing a decreased leaf N content, in line with the findings of Nakano et al. (1997). This is demonstrated in Figure 6.1, by the fact that the mass ratio of Rubisco to the thylakoid ATP-synthase does change, but simply as a function of leaf N content and independently of growth  $pC_a$  or N application. There is therefore no evidence for a direct mechanism for optimising the balance between carboxylation and RuBP-regeneration capacities in response to long-term growth at elevated  $\text{CO}_2$ . Makino et al. (1997) observed an increase in photosynthetic rate per unit leaf N in rice plants genetically manipulated to decrease Rubisco expression, measured under conditions of short-term exposure to elevated  $\text{CO}_2$ . In agreement with the relationship shown in Figure 6.6, they found the greatest advantage in leaves with high N content. The work presented here suggests that most of this advantage would be preserved where the plants were grown under conditions of elevated  $\text{CO}_2$ , since there is still excess investment in Rubisco under these conditions. There is therefore potential for improving the adaptation of crop plants to elevated  $\text{CO}_2$ , e.g. by genetic manipulation to decrease the amount of Rubisco relative to other photosynthetic components.

## 6.6 Critical appraisal of methodology and results

### 6.6.1 *Growth Environment*

For plants growing in the field, productivity is not controlled exclusively by any one environmental factor; temperature, water supply, atmospheric humidity, solar radiation and wind speed as well as soil factors, all affect the productivity of crops under field conditions and will interact with the effect of CO<sub>2</sub> (Lawlor and Mitchell, 1991). The results of an experiment on the effects of elevated CO<sub>2</sub> are therefore dependant on the particular conditions under which it took place. There are major differences in key factors, between controlled-environment (CE) chambers and the field: CE chambers are often hot, wet, humid and poorly illuminated, whereas in the field a crop may experience rapid changes in water, radiation and temperature. Given the above, a valid criticism of this study would be that the results obtained were not established in the field, and therefore how relevant are they to the natural environment?

Firstly, it is important to recognise that the experiments of this study were in fact done in a purpose built semi-controlled facility at Rothamsted (discussed in detail in Lawlor et al., (1993), and in section 2.1), the merits of which, aside from the control of  $pC_a$ , includes transmission of up to 70% of natural radiation and therefore the ability to track ambient photoperiod, a spectral quality very similar to sunlight over the range 400 to 700 nm (PAR), control of temperature to track outside ambient, and due to the >12 m<sup>3</sup> capacity of each walk-in chamber, the ability to grow large continuous crop stands. As such, the facility is a considerable advancement towards field conditions over conventional CE growth chambers, and only significantly different from OTCs (generally accepted as the next best thing to

the field given that few can afford the running costs for free air CO<sub>2</sub> enrichment (FACE)), in that crops cannot be sown directly into a limitless volume of soil for root growth, or be as readily subjected to airborne pests, disease, or precipitation (although compared to the field, OTCs will exert their own modifications on these factors anyway). Critically, in contrast to the variation obtained in the field, both OTCs and the semi-controlled facility at Rothamsted will be similar, in that humidity will often be higher, and windspeed either non-existent, or profiles severely modified.

Secondly, it is important to recognise that the methodology and growth of plants was done in such a way as to make experiments (and here we are primarily considering Experiments One and Three of this study - others to be discussed later), as field-like as possible. For example, it is recognised that a major deficiency of pot experiments is the typically small volume of soil available to roots, necessitating frequent watering and nutrient applications. This is compounded for experiments investigating the effects of growth at elevated  $pC_a$ , due to the potential for photosynthetic acclimation to occur, which typically is not observed in the field or pots of volume equal to or greater than 10 L (Sage, 1994; Medlyn, 1996), and is considered to be a response to sink-limitation via a restriction on root growth. For these reasons, 9 plants (representing spacings reflective of the field to give the equivalent of 255 plants m<sup>-2</sup>) were grown per 10 L pot, providing a depth of 300 mm for root growth. As discussed in detail in section 2.2, six pots were tightly packed into plastic boxes, modified with plastic sheeting to form a reservoir/artificial water table to prevent excess leaching of nutrients, whilst not compromising aerobic soil conditions for root growth. In turn, boxes were arranged into 3 x 4 continuous arrays of 2.9 m<sup>2</sup>, with attempts to minimise edge effects made

by hanging reflective silver screens from the top of canopies down to soil level around the perimeter of arrays, and rotating boxes within arrays on an approximate weekly basis. These precautions and approaches were all done to make experiments as field-like as possible. Additionally, whenever pots were removed for destructive harvests, a continuous crop canopy was maintained by decreasing the size of arrays and filling gaps (a positive advantage over the field and OTCs, as plants drilled into the soil clearly cannot be moved to fill gaps created by destructive harvests midway through experimentation). Further, where plants were subjected to different N treatments within an array, shorter plants were raised on wooden blocks so that potential shading effects were removed. The large capacity of pots also determined that nutrient applications were only necessary on a 7 to 10 d basis, which, coinciding with rotation within arrays and of arrays between chambers, meant that disruption to the growing crop stands was otherwise kept to a minimum.

Thus, through the use of the semi-controlled facility and these methods, Experiment One was as near field-like as possible, having tracked outside temperature and photoperiod, and been exposed to natural radiation with some artificial supplementation (summarised in Table 2.1). Likewise Experiment Three tracked ambient photoperiod, however natural radiation was screened out as a result of evidence from Experiment One that this most variable of factors may have resulted in apparent chamber effects for replicate treatments, despite rotation between chambers to try to minimise such an effect. This made Experiment Three less field-like than Experiment One, however mean radiation intensities were not dissimilar for the two experiments (Table 2.1).

Experiments Two and Four were never designed to be field-like, but rather to be totally controlled, with the aim of pushing plants to environmental extremes

(low N, 100 Pa CO<sub>2</sub>) to assess mechanisms and responses of gas exchange and biochemistry at the level of the leaf. The benefits of CE experiments to understand mechanisms has been justified and discussed elsewhere (Lawlor and Mitchell, 1991). It was considered that if no evidence for a mechanism of optimisation in the leaf in response to elevated CO<sub>2</sub> could be observed under extreme but stable CE conditions, then it was questionable whether such a response would ever be observed under more variable field-like conditions.

Another criticism of experimental approach might be with regard to the different N treatments applied in different experiments. From an agronomic perspective the criticism is accepted, as it would be unwise to directly compare biomass and yield values of Experiments One and Three, as the total amounts and way in which N was applied were different, and this has been addressed elsewhere (section 3.3, page 111). However, from a physiological/biochemical perspective (which was the main focus of the study), differences in N applications between experiments are of little consequence, as gas exchange parameters and activities/amounts of components were ultimately analysed and compared as a function of leaf N content. Further, from a whole plant perspective, absolute plant N content was not as important as the question of how growth at elevated CO<sub>2</sub> affected the way in which it was allocated into different organs.

With hindsight, one valuable modification to Experiment One in particular (as it was the most field-like), could have been the inclusion into experimental design of an equivalent N treated 'control' array of Minaret, sown into pots and boxes and screened as for chamber grown plants, but placed outside in the ambient environment, close to the glasshouse containing the semi-controlled facility. Whole plant morphological, physiological and biochemical responses could then have been

compared with 36 Pa CO<sub>2</sub> grown chamber plants, to validate directly any significant modifications of the chambers on plant responses, compared to those in the 'field'. The adoption of such 'control' plots has previously been used in the validation of OTC experiments (Mulholland et al., 1997; van Oijen et al., 1998), similarly investigating the effects of CO<sub>2</sub> enrichment.

Thus, having considered and discussed the growth environment, methodology and general approach, in the next section, we shall consider whether these factors worked together to make results for this study representative and comparable with results from the limited number of CO<sub>2</sub> enrichment studies done in the field.

#### 6.6.2 *Whole plant, physiological and biochemical responses*

Prior to making a comparison of results from this study with those of others, it is worth mentioning that in support of the methodology, in both Experiments One and Three, plants took up all the N that was applied (Fig. 2.7), suggesting that precautions to prevent leaching from pots was adequate, yet rooting conditions still suitably aerobic that uptake could occur.

Table 6.1 below compares the biomass and yield results from Experiments One and Three of this study, with those from various others, including the Arizona FACE experiments (Kimball et al., 1995; Garcia et al., 1998), OTC studies in Nottingham, UK (Mulholland et al., 1997) and Wageningen, Netherlands (van Oijen et al., 1998), which used the same Minaret cultivar as in this study, and results from previous experiments done in the Rothamsted semi-controlled facility (Mitchell et al., 1993; 1995; 1996). If we consider all experiments/treatments where N applied was > 20 g m<sup>-2</sup> (including 55 and 120 Pa CO<sub>2</sub> grown plants), then average biomass



and yield stimulation was 19 and 18% respectively (19 and 21% respectively when only 70 Pa CO<sub>2</sub> grown plants are considered). The results for high N grown plants from Experiments One and Three of this study agree well with these figures, suggesting that for biomass and yield, this study has been representative of the field, and the methodologies used validated. It is interesting to note that for the ten experiments reported for wheat (Table 6.1), the overall average increases of 19 and 18% respectively for biomass and yield with CO<sub>2</sub> enrichment, are considerably less than 31 and 35% respectively, given previously for wheat in the review of Cure and Acock (1986).

Phenological and morphological responses to long-term CO<sub>2</sub> enrichment for plants of Experiments One and Three have already been discussed in section 2.10 (pages 64-67) and compared in detail with other studies. However, to briefly summarise, both in Experiments One and Three of this study, and in the field-like OTC experiments of Mulholland et al., (1997) and van Oijen et al., (1998), all growing Minaret, long-term growth at approximately 70 Pa CO<sub>2</sub>, had no significant effect on phenological development, tiller density or canopy LAI. Further, in this study and that of Mulholland et al., (1997), growth at elevated CO<sub>2</sub> had no significant effect on mainstem leaf number, whilst van Oijen et al., (1998) report a 4 to 9% decrease, although it is unclear whether this was significant. Thus overall, responses observed in this study compare favourably for the same cultivar studied in OTCs.

Author	Year	Cultivar	Growth conditions and Treatments	Plant responses				
				Exposure facility	CO <sub>2</sub> Pa	Applied N (g m <sup>-2</sup> )	Positive biomass stimulation (%)	Positive yield stimulation (%)
Garcia et al., (1998)	1993/94	S. Wheat	Yecora Rojo	FACE	55	27	-	10
Wechsung et al., (2000)	1996	S. Wheat	Yecora Rojo	FACE	55	7	-	12
						35	-	15
Mulholland et al., (1998)	1995	S. Wheat	Minaret	OTC	55	20	-	10
					68	20	23	26
Theobald, this study	1995	S. Wheat	Minaret	Semi-controlled	70	4	6	6
					70	9	9	10
					70	24	18	19
Theobald, this study	1996	S. Wheat	Minaret	Semi-controlled	70	9	10	5
					70	22	21	16
Van Oijen et al., (1998)	1995/96	S. Wheat	Minaret	Cooled OTC	70	21	-	23
Mitchell et al., (1993)	1990/91	W. Wheat	Mercia	Semi-controlled	69	49	12	15
Mitchell et al., (1995)	1991/92	W. Wheat	Mercia	Semi-controlled	70	>30	27	39
Mitchell et al., (1996)	1993/94	W. Wheat	Mercia	Semi-controlled	69	>30	14	13
Havelka et al., (1984)	1982/83	W. Wheat	-	OTC	120	-	20	17

**Table 6.1** Summary of biomass and yield responses for spring and winter wheat grown at various  $pC_a$  and N regimes, but under as near field-like conditions as possible. Results are from various authors as indicated in the first column.

In this study, results from two experiments, indicate that growth at elevated CO<sub>2</sub> had limited effect on the partitioning of dry matter between organs. Exceptions were briefly at anthesis in Experiment Three when the fraction of dry mass allocated to leaves was 12 and 21% lower respectively for low and high N grown plants, and for the final harvest of Experiment One, when the fraction of dry mass allocated to stems was significantly higher at elevated CO<sub>2</sub>, and the fraction of dry matter allocated to chaff was surprisingly higher at 36 Pa CO<sub>2</sub> (presumably due to slower remobilisation and grain filling). However, CO<sub>2</sub> enrichment had no significant effect on the fraction of dry mass allocated to the grain (similar to harvest index (HI)) in either experiment. In support of this finding and the relevance of this study to the field, for spring wheat just prior to flowering and grown under FACE, root and stem masses were similarly stimulated by 23 and 21% respectively at 55 Pa CO<sub>2</sub> (Wechsung et al., 1995), suggesting no change in partitioning. Further, Mulholland et al., (1997) found no effect of CO<sub>2</sub> on HI for Minaret, and Havelka et al., (1984) also in an OTC study, observed no effect of CO<sub>2</sub> on the HI of winter wheat. In an experiment on winter wheat in which ambient UK conditions were tracked in a semi-controlled facility, Mitchell et al., (1993) found no significant effect of elevated CO<sub>2</sub> on the fraction of dry matter allocated to the grain for high N grown plants, although a decrease was observed for low N plants. The only other subtle effect that Mitchell et al., (1993) observed, was an increase in the fraction of dry matter in stems, as seen at final harvest in Experiment One of this study, and previously in spring wheat (Sionit et al., 1981).

In a comparative analysis of photosynthesis results from this study and others in which Minaret was grown at various sites across Europe in OTCs, it was found

that flag leaves post full emergence but prior to anthesis and of ample N treatment, measured at growth  $pC_a = 36$  Pa, gave comparable photosynthetic rates of on average  $25 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$  (Mitchell et al., 1999; Table 1, Fig. 1). Further, for all including this study, elevation to 70 Pa  $\text{CO}_2$  stimulated photosynthesis by approximately 48% (Mitchell et al., 1999; Fig. 1), this being in line with standard model predictions when there is no change in capacity (Farquhar and Sharkey, 1994). Comparably, for spring wheat grown in the field under FACE, average midday values of photosynthesis for 36 Pa  $\text{CO}_2$  grown flag leaves measured at a PPFD of 1200 to 2000  $\mu\text{mol m}^{-2} \text{ s}^{-1}$ , ranged from 17 to 24  $\mu\text{mol m}^{-2} \text{ s}^{-1}$ , with a stimulation of 28% at 55 Pa  $\text{CO}_2$ . Assuming a linear response, this would equate to a 50% stimulation of photosynthesis at 70 Pa  $\text{CO}_2$ , and equivalent to the 48% stimulation found above for this study and others in OTCs.

In the analysis of Mitchell et al., (1999), to determine whether any change in capacity was occurring through the season, the standard parameters  $\varepsilon$  and  $A_{\text{max}}$  were derived from  $A/pC_i$  curves (as discussed in Chapter 3 of this study). No evidence of a decrease in maximal capacity of leaves measured prior to anthesis for photosynthesis with  $\text{CO}_2$  enrichment was found (Mitchell et al., 1999; Fig.2), all studies analysed including the data from this study, being consistent in this respect. However, when treatment effects were followed throughout ontogeny, there was consistent evidence that the advantage of  $\text{CO}_2$  enrichment on photosynthesis prior to anthesis, declined virtually to zero during the grain filling period (Mitchell et al., (1999; Fig. 3), for all studies including this one.

Importantly parameters of the  $A/pC_i$  response were also followed through time, and in both this study, others analysed in Mitchell et al., (1999) and the FACE experiments of Nie et al., (1995a), there was evidence of a more rapid decline of  $\varepsilon$  at

elevated  $\text{CO}_2$  relative to  $A_{\text{max}}$ . Further, as shown in Chapter 3 of this study, there was an interaction of this response with N, occurring earlier in the low N treatments, and consistent with the findings of Rogers et al., (1996).

To determine for this study whether this observation was a true acclimation of the balance between carboxylation and RuBP-regeneration capacities to growth at elevated  $pC_a$ , parameters were analysed as a function of leaf N content (section 3.4). It was concluded that there was no evidence for a change in the *in vivo* balance between capacities determining photosynthesis in leaves of plants grown at 70 Pa  $\text{CO}_2$ , compared to those grown at 36 Pa  $\text{CO}_2$ . However, a recently re-modelled analysis of the same gas exchange data (Mitchell et al., 2000) has shown that contrary to the previous conclusion, there is in fact evidence for a specific effect of  $\text{CO}_2$  treatment, where carboxylation capacity is decreased more than RuBP-regeneration capacity for a given leaf N content. Important to note however, is that the acclimation of *in vivo* balance is only partial and mostly occurs in older leaves at low N supplies. Nevertheless the main conclusions of this study presented below in section 6.7 still hold true.

The observation of a partial acclimation of *in vivo* capacities to growth at elevated  $\text{CO}_2$  for this study, confirms the conclusions from a review of earlier studies (Sage, 1994), that the response is variable and more likely to occur when N is deficient. In a field experiment on wheat, Rogers et al., (1996) observed that Rubisco decreased (and assuming no compensatory increase in activation, the carboxylation capacity decreased also) more in response to elevated  $\text{CO}_2$  in plants grown with limiting N supply than with free access to N. Further, in the Arizona FACE experiments on spring wheat, no evidence was found for a decrease in photosynthetic capacity in young, fully expanded leaves until grain-fill in a well

fertilised crop (Garcia et al., 1998). In a controlled-environment experiment on wheat, Farage et al., (1998) demonstrated that changes in photosynthetic capacity at elevated CO<sub>2</sub> were completely eliminated by supplying N in proportion to growth, thus removing the indirect effect of CO<sub>2</sub> in increasing the limitation of N on growth. In a study specifically looking at the ratio of RuBP-regeneration capacity:carboxylation capacity in soybean (Sims et al., 1998), growth at elevated  $pC_a$  increased the ratio more in plants grown at low N than at high N. Further, they found that low N supply alone either had no effect or slightly increased this ratio. This is in contrast to the findings of Mitchell et al., (2000) following reanalysis of the gas exchange data of this study, where low N altered the ratio between carboxylation and RuBP-regeneration capacities in favour of the latter, a view consistent with the observation that the ratio of Rubisco:ATP-synthase contents increases in wheat leaves with high N contents (Theobald et al., 1998). However, irrespective of the direction of *in vivo* acclimation of capacities, consistently the response is only partial and typically occurs late in leaf ontogeny, and this is observed in both semi-controlled experiments, through to those done in the field.

Few studies have comprehensively investigated photosynthetic and component responses to long-term growth at elevated CO<sub>2</sub> of various leaves down a vertical canopy profile, with the exception of the Arizona FACE experiments (Osborne et al., 1998) and this study, in both of which flag, flag-1 and flag-2 leaves were focussed on. Consistently for both studies, CO<sub>2</sub> enrichment had no significant effect on  $\epsilon$ ,  $V_{c,max}$ ,  $A_{max}$ , leaf N or Rubisco content in any leaf in the canopy prior to anthesis. Further, CO<sub>2</sub> enrichment had no subsequent effect on any parameter in flag-2 leaves of this study. However, for all other leaves of both studies, CO<sub>2</sub> enrichment caused significant decreases in all parameters and components during

the period of grain-fill, so that normal ontogenic remobilisation of resources occurred fastest in the order of flag-2, flag-1 and then flag leaves, with the enhancement effect of CO<sub>2</sub> superimposed upon this, so that leaf senescence in elevated CO<sub>2</sub> grown plants finished sooner.

Also consistent in both studies, were the observations that  $\epsilon$ ,  $V_{c,max}$ ,  $A_{max}$ , Rubisco and additionally in this study, ATP-synthase, Chl and soluble protein, were always highly correlated with leaf N content, and there was no effect of elevated CO<sub>2</sub>, leaf age or position on these relationships (Osborne et al., 1998; this study sections 4.6.4 and 5.4.4). That is, decreases in parameters of photosynthesis or leaf components, were directly in proportion to the decrease in leaf N, in both studies, so that elevated CO<sub>2</sub> did not decrease the proportion of N allocated to Rubisco within leaves.

One previously proposed mechanism to explain acclimation and the faster loss of capacity and components from leaves growing at elevated  $pC_a$ , has been the suggestion that sugar accumulation within leaves can lead to the suppression of photosynthetic gene expression (van Oosten and Besford, 1996), although this will typically apply to all photosynthetic genes (Krapp and Stitt, 1995). However, in the later leaves of wheat, this mechanism seems less likely as fructan synthesis in the internodes represents a very large sink for sugar export. This is further supported, again consistently, in both this study and the FACE experiments, where leaf carbohydrate contents were not significantly increased under elevated  $pC_a$  (Nie et al., 1995b; this study Fig. 2.9 and associated text, section 3.6). Further, Nie et al., (1995b), found no correlation between carbohydrate status and message levels for photosynthetic components in wheat under FACE.

However, an explanation which can account for the observations made in this study, a number of OTC chamber experiments done throughout Europe (Mitchell et al., 1999), and the Arizona FACE experiments with wheat, is that the timing of events are consistent with an increased demand for N from an increased number of grain at elevated  $pC_a$ . For example, from the FACE experiments on wheat, Osborne et al., (1998) report that although there was a slight 3% decrease in the fraction of N in individual grains, the absolute quantity of N in the grain at harvest was 5% higher, because of an 8% increase in grain yield at elevated  $pCO_2$ . Since total plant N content was unchanged at elevated  $pCO_2$ , increased mobilisation from other plant parts must have occurred to support the increased amount of N allocated to grain. Thus, these results can be explained in terms of the effects that growth at elevated  $CO_2$  has on source-sink balance in the crop. Before grain-fill, the extra C assimilated at elevated  $pC_a$  can be used in ear growth or stored as fructans in the internodes. During grain-fill however, the growing grains will be a finite sink for C, and are also the final destination for the majority of leaf N. Elevated  $CO_2$  may increase the number of grains and therefore the size of the sink, but this will also increase demand for N re-mobilisation from the leaves. If source of C from the leaves exceeds demand from the grains, or if there is increased demand for N, an increase in the rate of decline of photosynthetic apparatus can result (Evans, 1993). Therefore, there is no requirement to invoke a direct response of capacity to  $CO_2$  concentration to explain the consistent results in this and other studies (Mitchell et al., 1999), since a source-sink explanation is consistent with the finding that the relative decline in capacity at elevated  $CO_2$  compared with ambient is gradual, and particularly in this study, greater in low N regimes.



From the comparative discussion above, the implication is that the methodology and general approach discussed in 6.6.1 and used in this study, did achieve the task of making Experiments One and Three as field-like as possible, since so many absolute values for various whole plant, morphological, physiological and biochemical parameters consistently match those obtained in the field and OTCs. Further, patterns of response, e.g. the decline of components within leaves and between different leaves within the canopy, are equally consistent between the semi-controlled environment and the field. Thus, I consider that the results and observations of this study are relevant to the field, both now and for future growth conditions. All that remains in the final section of this appraisal, is to briefly put the findings into context.

### 6.6.3 *Challenges for the future*

The observations of this study show that under conditions similar to those in the UK, increasing CO<sub>2</sub> concentration may be expected to increase total dry matter production and grain yield of spring wheat, even under limiting N conditions. However, in general, there is no evidence, not even under severely limiting N supply, for a substantial re-distribution of N at elevated  $pC_a$ , either at crop level (i.e. away from leaves to other components) or within the leaf (i.e. away from Rubisco to other components determining RuBP-regeneration), suggesting that spring wheat does not respond to elevated  $pC_a$  in the optimal manner to maximise yield. Within leaves, this means that for a doubling of  $pC_a$  to 70 Pa, Rubisco may be in excess by up to 30% representing an approximate 6 to 7% of total leaf N.

Speculatively, if during the early development of leaves, this excess N were remobilised to other components, particularly those determining RuBP-regeneration

capacity, then even higher rates of photosynthesis may be sustained (assuming sufficient light and translocation of assimilate from leaves) under elevated CO<sub>2</sub>. This could lead to a still greater accumulation and storage of carbohydrate in stems or elsewhere during vegetative stages of growth (the suggestion from this study and others being that wheat has the capacity to do this), which in turn could further increase yields by supporting the survival of more ear bearing tillers, with larger ears containing more grain. Alternatively, if the excess N in leaves were remobilised towards in particular, the roots, then this could result in increased nutrient foraging and uptake. For wheat growing at elevated CO<sub>2</sub>, the need for increased nutrient uptake could become particularly important, if, despite increased grain yields, the proportion of N allocated to individual grains, and therefore their nutritional value, decreases, as found in this study (Table 2.11) and those of others (Osborne et al., 1998). Alternatively, once mechanisms are fully understood, attempts could be made to engineer or breed by conventional means, plants that are able to re-mobilise a greater proportion of total N that they contain to the grain by physiological maturity. For example, in Experiments One and Three of this study, growth at 70 Pa CO<sub>2</sub> had no significant effect on the fraction of total crop N distributed to the grain at final harvest, some 15 to 30% remaining in roots and straw, representing a possible target for future improvement.

What is clear from this study, is that there is scope for improving the N-use efficiency of wheat under elevated CO<sub>2</sub> conditions, although as a note of caution, success, as demonstrated by the results of others, is by no means guaranteed. In the Rubisco antisense studies with the tobacco model system, a decrease in Rubisco by antisense *rbtS* had little effect on chlorophyll content or other components of photosynthesis (Andrews et al., 1995; Stitt and Schulze, 1994), and therefore it is not

clear whether N re-allocation from Rubisco to other components occurred. Indeed, decrease in Rubisco never led to higher rates of photosynthesis under conditions of CO<sub>2</sub> enrichment (Quick et al., 1991; von Caemmerer et al., 1994). However, the nitrate pool in tobacco plants is typically large, and in the antisense plants was greater than the decrease in N allocated to Rubisco (Fichtner et al., 1993; Masle et al., 1993), suggesting that the re-mobilised N was compensated for by increased free-nitrate within leaves.

In contrast, Makino et al., (2000), report more success with Rubisco-antisense rice plants. At 100 Pa CO<sub>2</sub>, antisense plants with 65% of wild-type Rubisco showed 5-15% higher rates of photosynthesis than the wild-type plants for the same leaf N content. This increase was accompanied by a 5-15% greater content of Chl, cytochrome *f* and coupling factor 1 at the same leaf N content (Makino et al., 1997). In addition, these incremental ratios exactly corresponded to the ratio of decreased fraction of leaf N present as Rubisco. Thus, unlike tobacco, in rice N seems to have been optimally allocated between Rubisco and components limiting CO<sub>2</sub>-saturated photosynthesis. Following this evidence that selected antisense plants had a higher N-use efficiency at the leaf level, attention was directed to whether such transgenic plants could grow more adaptively in elevated CO<sub>2</sub> environments. Disappointingly, when plants were grown for 70 days at 100 Pa CO<sub>2</sub>, there was no significant difference in total biomass between the antisense and wild-type plants, shoot/root ratios also being similar. Thus it was concluded that a higher N-use efficiency at leaf level does not necessarily lead to greater production of biomass, but can enhance N-use efficiency of the whole plant.

Finally, the variability of the natural environment has to be considered. Whilst theory suggests that optimal N-use efficiency may occur in leaves at 70 Pa

CO<sub>2</sub> via a 30% decrease in the amount of Rubisco, followed by re-allocation of the N contained within, theory is typically based on the assumption of constant and stable environments. It is necessary to remember that photosynthetic components are often not optimised such as to maximise photosynthesis for (unnatural) constant conditions, but rather appear to invest such that they can exploit changes that would be expected in a natural environment (Stitt and Schulze, 1994). Given that a further consequence of increasing CO<sub>2</sub> and other greenhouse gas emissions could be a 2-3°C rise in mean global temperature by 2100, with possible other changes in climate such as humidity and cloud cover (variables not investigated in this study), a 30% decrease in wheat Rubisco by antisense, whilst it may be beneficial to PNUE, may not consistently give biomass and yield advantage in higher CO<sub>2</sub> environments in combination with other factors. For this reason, if, and hopefully when, attempts to decrease wheat Rubisco by antisense are successful, plants will nevertheless have to be rigorously validated for a combination of environmental situations to determine whether advantage will still be maintained.

## 6.7 Summary of results

### 6.7.1 *Whole plant responses to long-term growth at elevated $pC_a$*

For results obtained from Experiment One, growth at elevated  $pC_a$  did not affect phenology or mainstem appearance, but did increase biomass by 6, 9 and 18% respectively for low (4 g m<sup>-2</sup>), medium (9 g m<sup>-2</sup>) and high (24 g m<sup>-2</sup>) N grown plants, CO<sub>2</sub> and N having a positive interaction ( $P < 0.05$ ). Grain yield was affected in a similar manner, with CO<sub>2</sub> stimulations of 6, 10 and 19% for low, medium and high

N treatments respectively, this effect being due almost entirely to an increased number of grains per ear. Total N uptake by crops was unaffected by  $pC_a$ . There was no consistent increase in the fraction of dry mass in roots at elevated  $pC_a$  and low N, or decrease in the fraction of crop N in green leaves at elevated  $pC_a$  prior to anthesis, but during grain fill the amount of Rubisco and N in flag leaves declined slightly faster at elevated  $pC_a$ . However, the fraction of crop N in the grain was not significantly greater for plants grown at elevated  $pC_a$  at final harvest.

Therefore, from these results, no evidence has been obtained to support the specific hypothesis being tested at the level of the whole plant, that growth of *Triticum aestivum* L. cv Minaret at 70 Pa CO<sub>2</sub> and particularly at low N, should allow for a decrease in N investment in leaves to release N for the growth of roots and/or reproductive organs to utilise the increased fluxes of carbohydrate that prevail under elevated  $pC_a$  conditions. However, since such a response is only expected to be mediated via gene suppression under conditions of a source-sink imbalance, this suggests that an imbalance did not occur, reasons for which have been discussed previously (section 2.10).

#### 6.7.2 *Response of the in vivo balance of capacities determining photosynthetic rate to long-term growth at elevated $pC_a$ , determined from gas exchange analysis*

For a doubling of  $pC_a$  to 70 Pa, a 30 to 40% increase in the ratio of light-saturated RuBP-regeneration capacity to carboxylation capacity has been predicted for optimal N-use efficiency (Webber et al., 1996; Medlyn, 1996). To test this hypothesis the shape of  $675 A/pC_i$  curves from four experiments were analysed for a CO<sub>2</sub> stimulation effect, calculated from the ratio of photosynthetic rates obtained at  $pC_a = 36$  ( $A_{36}$ ) and 70 ( $A_{70}$ ) Pa CO<sub>2</sub>, an increase from 1.4 at current  $pC_a$  to a

hypothetical (Sage, 1994) 1.8 at  $pC_a=70$  Pa being expected if optimal rebalancing between capacities occurs.

For the CO<sub>2</sub> stimulation ratio as a function of time, and for leaves of various insertion, a general pattern emerged (Fig. 3.6) in which at full expansion no significant effect of growth at elevated CO<sub>2</sub> was observed in any leaf of any experiment, so that a ratio of 1.4 persisted, indicating no change to the *in vivo* balance between carboxylation and RuBP-regeneration capacities. Thereafter and particularly for flag leaves, significant ( $P<0.001$  to  $P<0.05$ ) CO<sub>2</sub> effects on stimulation were observed from 9 to 16 d post-anthesis, with the stimulation being greatest for low N grown plants, and the timing of response coinciding with grain filling. Thus initially, these results suggested that there was a direct CO<sub>2</sub> induced change on the *in vivo* balance of capacities in a direction in accord with the hypothesis being tested. However, as observed ratios were never greater than 1.6, this suggested that only a partial re-balancing was occurring, and the late timing of the response in ontogeny questions the benefits to be gained.

Further, when CO<sub>2</sub> stimulation responses were plotted as a function of leaf N content (Fig. 3.10), responses for all experiments were found to be independent of both growth CO<sub>2</sub> and N treatment. However, there was some evidence to suggest that the CO<sub>2</sub> stimulation ratio may actually increase solely as a function of leaf N content and independent of CO<sub>2</sub>, supporting previous observations of Nakano et al., (1997). Therefore, since apparent effects of elevated CO<sub>2</sub> on the CO<sub>2</sub> stimulation ratio can be explained simply via a decrease in leaf N content, in conclusion, no evidence was found from gas exchange analysis, to support the hypothesis in this study for a 30 to 40% shift in the *in vivo* balance between carboxylation and RuBP-regeneration capacities for plants grown at 70 Pa CO<sub>2</sub>.

To determine whether any changes to the *in vivo* balance between capacities for RuBP-regeneration and  $\text{PO}_4^{3-}$ -regeneration were occurring at elevated  $pC_a$ , the  $\text{O}_2$  sensitivity response of photosynthesis at various  $pC_a$  was determined, for various leaves through time (Fig. 3.7). With the exception of two occasions in different experiments, plants always showed  $\text{O}_2$  sensitivity, indicating that the capacity to export triose phosphate from chloroplasts and subsequently regenerate  $\text{PO}_4^{3-}$  was never completely limiting. When  $\text{O}_2$  sensitivity responses were plotted as a function of leaf N content (Fig. 3.11), the relationships were found to be independent of both growth  $\text{CO}_2$  and N treatment, with the mean stimulation for photosynthesis measured at  $pC_a=70$  on decreasing the  $\text{O}_2$  partial pressure from 21 to 2% averaging 15%. Therefore, these results support the contention that there was still potential for improving the *in vivo* balance between carboxylation and RuBP-regeneration capacities at 70 Pa  $\text{CO}_2$ .

### 6.7.3 *Response of components of the photosynthetic machinery to long-term growth at elevated $pC_a$*

Despite the lack of evidence from gas exchange analysis to support an *in vivo* rebalancing of capacities at elevated  $\text{CO}_2$ , since gas exchange measurements only reflect the activity of components, the possibility that changes to the amounts of components had occurred but were masked by altered activation could not be ruled out. Therefore to test this, and the specific hypothesis of whether growth at elevated 70 Pa  $\text{CO}_2$  would allow for optimal N allocation within the photosynthetic machinery, mediated via a specific decrease of 30 to 40% in the amount of Rubisco relative to the thylakoid ATP-synthase (chosen as the determinant of RuBP-regeneration capacity), measurements on these components were made.

From the results of three separate experiments, in which leaf samples were taken from full emergence through to senescence, long-term growth at 70 Pa CO<sub>2</sub> had no significant effect on the amounts of Rubisco or leaf N prior to at least six days after anthesis. Thereafter, some significant effects between the two CO<sub>2</sub> treatments were observed, with amounts of Rubisco and N being decreased in the 70 Pa CO<sub>2</sub> treatment. However, when the amount of Rubisco was plotted as a function of leaf N content, the relationship was found to be independent of growth CO<sub>2</sub> and N treatment, demonstrating that there was no CO<sub>2</sub>-specific decrease in Rubisco as a proportion of leaf N content.

Similarly, growth at elevated  $pC_a$  had no significant effect on amounts of the thylakoid ATP-synthase in leaves, in any treatment of any occasion. Prior to at least six days after anthesis, the same was also generally true for leaf N content, but thereafter some significant effects of the CO<sub>2</sub> treatments were observed, with amounts of N being lower in the 70 Pa CO<sub>2</sub> grown plants. However, when the amount of thylakoid ATP-synthase was plotted as a function of leaf N content, the response as for Rubisco, was independent of growth CO<sub>2</sub> and N treatment. Thus there was no evidence for an increase in the amount of thylakoid ATP-synthase as a function of leaf N (the hypothesis being specifically tested given no evidence for a specific decrease in Rubisco).

Thus, when the responses of both of these components are taken together, we can conclude that there was no evidence in this study to suggest that a reallocation of N away from the main component of the least limiting capacity of carboxylation (Rubisco), to one of the main components of the limiting capacity of RuBP regeneration (thylakoid ATP-synthase), took place in plants grown at 70 Pa CO<sub>2</sub> and either low or high N applications. This conclusion is despite a 17% decrease in the



initial activity of Rubisco in plants shortly after full leaf expansion (grown and sampled at 100 Pa CO<sub>2</sub> and saturating light), suggesting that carboxylation capacity was excessive of requirements, so that some reallocation of resources could have taken place to increase N-use efficiency.

## 6.8 Study Conclusions

In this study, no evidence has been found to support a substantial redistribution of N at 70 Pa CO<sub>2</sub>, either at the whole crop level (i.e. away from leaves to other organs) or within the leaf (i.e. away from Rubisco to the capacity for RuBP-regeneration), even under conditions of severely limiting N supply. Therefore for leaves, there is no evidence of a direct mechanism for optimising the balance between carboxylation and RuBP-regeneration capacities in response to long-term growth at elevated CO<sub>2</sub>, suggesting that spring wheat does not achieve optimal N-use efficiency under these conditions. This is supported by the demonstration that there is excess investment in Rubisco over that required for maintaining  $A$  at elevated CO<sub>2</sub>, and that this excess is greater at high N. Therefore there is potential, through the genetic manipulation of the amount of Rubisco, for improving the N-use efficiency of spring wheat for growth in elevated CO<sub>2</sub> environments of the future, with the greatest advantage expected in leaves with high N content.



## Literature Cited

- Anderson JM, Osmond CB** (1987) Sun-shade responses: Compromises between acclimation and photoinhibition. *In* DJ Kyle, CB Osmond, CJ Arntzen, eds, Photoinhibition. Elsevier, Amsterdam, pp 1-39
- Andrews TJ, Hudson GS, Mate CJ, von Caemmerer S, Evans JR, Arvidsson YBC** (1995) Rubisco: the consequences of altering its expression and activation in transgenic plants. *J Exp Bot* **46**: 1293-1300
- Akazawa T** (1978) Structure and function of ribulose-1,5-bisphosphate carboxylase. *In* DO Hall, J Coombs, TW Goodwin, eds, Photosynthesis. Biochemical Society, London, Vol 77, pp 447-456
- Arp WJ** (1991) Effects of source-sink relations on photosynthetic acclimation to elevated CO<sub>2</sub>. *Plant Cell Environ* **14**: 869-876
- Arp WJ, Drake BG** (1991) Increased photosynthetic capacity of *Scirpus olneyi* after 4 years exposure to elevated CO<sub>2</sub>. *Plant Cell Environ* **14**: 1003-1006
- Arnon DI** (1949) Copper enzymes in isolated chloroplasts. Phenol oxidase in *Beta vulgaris*. *Plant Physiol* **24**: 1-15
- Azcon-Bieto J** (1983) Inhibition of photosynthesis by carbohydrates in wheat leaves. *Plant Physiol* **73**: 681-686
- Badger MR, Sharkey TD, von Caemmerer S** (1984) The relationship between steady state gas exchange of bean leaves and the levels of carbon-reduction-cycle intermediates. *Planta* **160**: 305-313
- Barnola JM, Raynaud D, Korotkevich YS, Lorius C** (1987) Vostok Ice Core Provides 160,000-Year Record Of Atmospheric CO<sub>2</sub>. *Nature* **329**: 408-14
- Besford RT** (1991) The Greenhouse effect. Acclimation of tomato plants growing in high CO<sub>2</sub>, relative changes in Calvin cycle enzymes. *Journal of Plant Physiol* **136**: 458-463
- Besford RT, Ludwig LJ, Withers AC** (1990) The greenhouse effect: Acclimation of tomato plants growing in high CO<sub>2</sub>, photosynthesis and ribulose-1,5-bisphosphate carboxylase protein. *J Exp Bot* **41**: 925-931
- Beynon RJ, Oliver S** (1996) Avoidance of proteolysis in extracts. *In* S Doonan, ed, Methods in Molecular Biology: Protein Purification Protocols, Vol 59. Humana Press Inc, Totowa, NJ, pp 81-93
- Björkman O** (1981) responses to different quantum flux densities. *In* OL Lange, PS Nobel, CB Osmond, H Ziegler, eds, Physiological plant ecology I. Responses to the physical environment. Springer-Verlag, Berlin, pp 57-107

- Bloom AJ, Chapin FS III, Mooney HA** (1985) Resource limitation in plants – an economic analogy. *Annu Rev Ecol Syst* **16**: 363-392
- Bowes G** (1993) Facing the inevitable: Plants and increasing atmospheric CO<sub>2</sub>. *Annu Rev Plant Physiol Plant Mol Biol* **44**: 309-332
- Bradford MM** (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilising the principle of dye-binding. *Anal Biochem* **72**: 248-254
- Brooks A, Farquhar GD** (1985) Effect of temperature on the CO<sub>2</sub>/O<sub>2</sub> specificity of ribulose-1,5-bisphosphate carboxylase oxygenase and the rate of respiration in the light-estimates from gas exchange measurements on spinach. *Planta* **165**: 397-406
- Bunce JA** (1993) Effects of doubled atmospheric carbon dioxide concentration on the responses of assimilation and conductance to humidity. *Plant Cell Environ* **16**: 189-197
- Butz ND, Sharkey TD** (1989) Activity of ribulose bisphosphate carboxylase accurately reflect carbamylation ratios. *Plant Physiol* **89**: 735-739
- Byrd GT, Brown RH** (1989) Environmental effects on photorespiration of C<sub>3</sub>-C<sub>4</sub> species. I. Influence of CO<sub>2</sub> and O<sub>2</sub> during growth on photorespiratory characteristics and leaf anatomy. *Plant Physiol* **90**: 1022-1028
- Cave G, Tolley LC, Strain BR** (1981) Effect of carbon dioxide enrichment on chlorophyll content, starch content and starch grain structure in *Trifolium subterraneum* leaves. *Physiol Plant* **51**: 171-174
- Conroy J, Hocking P** (1993) Nitrogen nutrition of C<sub>3</sub> plants at elevated atmospheric CO<sub>2</sub> concentrations. *Physiol Plant* **89**(3): 570-576
- Cook MG, Evans LT** (1983) Nutrient responses of seedlings of wild and cultivated *Oryza* species. *Field Crops Res* **6**: 205-218
- Cure JD, Acock B** (1986) Crop responses to carbon dioxide doubling: a literature survey. *Agric For Meteorol* **38**: 127-145
- Cure JD, Israel DW, Rufty TWJ** (1988) Nitrogen stress effects on growth and seed yield of non-nodulated soybean exposed to elevated carbon dioxide. *Crop Sci* **28**: 671-677
- Curtis PS** (1996) A meta-analysis of leaf gas exchange and nitrogen in trees grown under elevated CO<sub>2</sub> in situ. *Plant Cell Environ* **19**: 127-137
- Dalton CC** (1984) The effect of sucrose supply rate on photosynthetic development of *Ocimum basilicum* (Sweet Basil) cells in continuous culture. *J Exp Bot* **35**: 505-516

- Delgado E, Mitchell RAC, Parry MAJ, Driscoll SP, Mitchell VJ, Lawlor DW** (1994) Interacting effects of CO<sub>2</sub> concentration, temperature and nitrogen supply on the photosynthesis and composition of winter-wheat leaves. *Plant Cell Environ* **17**: 1205-1213
- Delucia EH, Sasek TW, Strain BR** (1985) Photosynthetic inhibition after long-term exposure to elevated levels of atmospheric carbon dioxide. *Photosynth Res* **7**: 175-184
- Dickinson C, Altabella T, Chrispeels M** (1991) Slow growth phenotype of transgenic tomato expressing apoplasmic invertase. *Plant Physiol* **95**: 420-425
- Drake BG, Gonzalez-Meler MA, Long SP** (1997) More efficient plants: a consequence of rising atmospheric CO<sub>2</sub>? *Annu Rev Plant Physiol Mol Biol* **48**: 609-639
- Edelmann J, Hanson AD** (1971) Sucrose suppression of chlorophyll synthesis in carrot cultures. *Planta* **98**: 150-156
- Ehleringer JR** (1989) Temperature and energy budgets. In RW Pearcy, JR Ehleringer, HA Mooney, PW Rundel, eds, *Plant physiological ecology. Field methods and instrumentation*. Chapman and Hall, London, pp 117-135
- Evans JR** (1983) Nitrogen and photosynthesis in flag leaves of wheat (*Triticum aestivum* L.). *Plant Physiol* **72**: 297-302
- Evans JR** (1985) A comparison of photosynthetic properties of flag leaves from *Triticum aestivum* and *T. monococcum*. In B Jeffcoat, AF Hawkins, AD Stead, eds, *Regulation of sources and sinks in crop plants*. British Plant Growth Regulator Group, Bristol, pp 111-125
- Evans JR** (1986) The relationship between CO<sub>2</sub>-limited photosynthetic rate and ribulose-bisphosphate-carboxylase content in two nuclear cytoplasm substitution lines of wheat and the co-ordination of ribulose-bisphosphate-carboxylation and electron transport capacities. *Planta* **167**: 351-358
- Evans JR** (1987) The relationship between electron transport components and photosynthetic capacity in pea leaves grown at different irradiances. *Aust J Plant Physiol* **14**: 157-170
- Evans JR** (1989) Photosynthesis and nitrogen relationships in leaves of C<sub>3</sub> plants. *Oecologia* **78**: 9-19
- Evans JR** (1993) Photosynthetic acclimation and nitrogen partitioning within a Lucerne canopy. I. Canopy characteristics. *Aust J Plant Physiol* **20**: 55-67
- Evans JR, Austin RB** (1986) The specific activity of ribulose-1,5-bisphosphate carboxylase in relation to genotype in wheat. *Planta* **167**: 344-350

- Evans JR, Seemann JR** (1984) Differences between wheat genotypes in specific activity of ribulose-1,5-bisphosphate carboxylase and the relationship to photosynthesis. *Plant Physiol* **74**: 759-765
- Evans JR, Seemann JR** (1989) The allocation of protein nitrogen in the photosynthetic apparatus: costs, consequences and control. *In* W Briggs, ed, *Photosynthesis*. Alan R Liss, New York, pp 183-205
- Evans JR, Terashima I** (1987) Effects of nitrogen nutrition on electron transport components and photosynthesis in spinach. *Aust J Plant Physiol* **14**: 59-68
- Evans JR, von Caemmerer S, Setchell BA, Hudson GS** (1994) The relationship between CO<sub>2</sub> transfer conductance and leaf anatomy in transgenic tobacco with a reduced content of Rubisco. *Aust J Plant Physiol* **21**: 475-495
- Farquhar GD, von Caemmerer S** (1982) Modelling of photosynthetic response to environmental conditions. *In* OL Lange, PS Nobel, CB Osmond, H Ziegler, eds, *Physiological Plant Ecology II. Water relations and carbon assimilation*, Vol 12B. Springer-Verlag, Berlin, pp 550-587
- Farquhar GD, von Caemmerer S, Berry JA** (1980) A biochemical model of photosynthetic CO<sub>2</sub> assimilation in leaves of C<sub>3</sub> species. *Planta* **149**: 78-90
- Farquhar GD, Richards RA** (1984) Isotopic composition of plant carbon correlates with water-use efficiency of wheat genotypes. *Aust J Plant Physiol* **11**: 539-552
- Farquhar GD, Sharkey TD** (1982) Stomatal conductance and photosynthesis. *Annu rev Plant Physiol* **33**: 317-345
- Farquhar GD, Sharkey TD** (1994) Photosynthesis and carbon assimilation. *In* KJ Boote, JM Bennett, TR Sinclair, GM Paulson, eds, *Physiology and determination of crop yield*. American Society of Agronomy, Madison, WI, pp 187-210
- Fichtner K, Quick WP, Schulze ED, Mooney HA, Rodermel SR, Bogorad L, Stitt M** (1993) Decreased ribulose-1,5-bisphosphate carboxylase-oxygenase in transgenic tobacco transformed with 'antisense' *RbcS*. V. Relationship between photosynthetic rate, storage strategy, biomass allocation and vegetative plant growth at three different nitrogen supplies. *Planta* **190**: 1-9
- Field C, Mooney HA** (1986) The photosynthesis-nitrogen relationship in wild plants. *In* TA Givinish, ed, *On the economy of plant form and function*. Cambridge University Press, London, pp 25-55
- Foyer CH** (1987) The basis of source-sink interactions in leaves. *Plant Physiol and Biochem* **25**: 649-657
- Fromme P, Graber P** (1989) Heterogeneity of ATP-hydrolysing sites on reconstituted CF<sub>0</sub>CF<sub>1</sub>. *FEBS Lett* **259**: 33-36

- Garcia RL, Long SP, Wall GW, Osborne CP, Kimball BA, Nie GY, Pinter PJ, LaMorte RL, Wechsung F (1998)** Photosynthesis and conductance of spring wheat leaves: field response to continuous free-air atmospheric CO<sub>2</sub> enrichment. *Plant Cell Environ* **21**: 659-669
- Goldschmidt EE, Huber SC (1992)** Regulation of photosynthesis by end-product accumulation in leaves of plants storing starch, sucrose, and hexose sugars. *Plant Physiol* **99**: 1443-1448
- Graham IA, Denby KJ, Leaver CJ (1994)** Carbon catabolite repression regulates glyoxylate cycle gene expression in cucumber. *Plant Cell* **6**: 761-772
- Graves J, Reavey D (1996)** The atmosphere of the earth, past, present and future. *In* Global environmental change. Longman, Harlow, pp 1-22
- Gunderson CA, Norby RJ, Wullschlegel SD (1993)** Foliar gas exchange responses of two deciduous hardwoods during 3 years of growth at elevated CO<sub>2</sub>: No loss of photosynthetic enhancement. *Plant Cell Environ* **16**(7): 797-807
- Gutteridge S, Parry MAJ, Schmidt CNG (1982)** The reactions between active and inactive forms of wheat ribulosebiphosphate carboxylase and effectors. *Eur J Bioch* **126**: 597-602
- Gutteridge S, Parry MAJ, Burton S, Keys AJ, Mudd A, Feeney J, Servaites J, Pierce J (1986)** A nocturnal inhibitor of carboxylation in leaves. *Nature* **324**: 274-276
- Habash DZ, Paul MJ, Parry MAJ, Keys AJ, Lawlor DW (1995)** Increased capacity for photosynthesis in wheat grown at elevated CO<sub>2</sub> – the relationship between electron-transport and carbon metabolism. *Planta* **197**: 482-489
- Harley PC, Sharkey TD (1991)** An improved model of C<sub>3</sub> photosynthesis at high CO<sub>2</sub>: reversed O<sub>2</sub> sensitivity explained by a lack of glycerate re-entry into the chloroplast. *Photosyn Res* **27**: 169-178
- Harris GC, Cheesbrough JK, Walker DA (1983)** Effects of mannose on photosynthetic gas exchange in spinach leaf discs. *Plant Physiol* **71**: 108-111
- Havelka UD, Wittenbach VA, Boyle MG (1984)** CO<sub>2</sub> –enrichment effects on wheat yield and physiology. *Crop Sci* **24**: 1163-1168
- Heber U, Niemanis S, Dietz KJ (1988)** Fractional control of photosynthesis by the Q<sub>B</sub> protein, the cytochrome *b/f* complex, and other components of the photosynthetic apparatus. *Planta* **173**: 267-274
- Heldt HW, Chon CJ, Maronde D, Herold A, Stankovic ZS, Walker DA, Kraminer A, Kirk MR, Heber U (1977)** Role of orthophosphate and other factors in the regulation of starch formation in leaves and isolated chloroplasts. *Plant Physiol* **59**: 1146-1155

- Helmerhorst E, Stokes GB** (1980) Microcentrifuge desalting: a rapid quantitative method for desalting small amounts of protein. *Analytical Biochem* **104**: 130-135
- Herold A** (1980) Regulation of photosynthesis by sink activity: the missing link. *New Phytol* **86**: 131-144
- Hicklenton PR, Jolliffe PA** (1980) Alteration in the physiology of CO<sub>2</sub> exchange in tomato plants grown in CO<sub>2</sub>-enriched atmospheres. *Can J Bot* **58**: 2181-2189
- Howe CJ, Bowman CM, Dyer TA, Gray JC** (1982) Localisation of wheat chloroplast genes for the beta and epsilon subunits of ATP synthase. *Mol Gen Genet* **186**: 525-530
- Huber SC, Hanson KR** (1992) Carbon partitioning and growth of starchless mutant of *Nicotiana sylvestris*. *Plant Physiol* **99**: 1449-1454
- Hudson GS, Evans JR, von Caemmerer S, Arvidsson YBC, Andrews TJ** (1992) Reduction of ribulose-1,5-bisphosphate carboxylase/oxygenase content by antisense RNA reduces photosynthesis in transgenic tobacco plants. *Plant Physiol* **98**: 294-302
- Jacob J, Greitner C, Drake BG** (1995) Acclimation of photosynthesis in relation to Rubisco and non-structural carbohydrate contents. *Plant Cell Environ* **18**: 875-884
- Jang JC, Sheen S** (1994) Sugar sensing in higher plants. *Plant Cell* **6**: 1665-1679
- Jones MGK, Outlaw WH, Lowry OH** (1977) Procedure for assay of sucrose in the range 10<sup>-1</sup> – 10<sup>-14</sup> moles. *Plant Physiol* **60**: 379-383
- Kacser H, Burns JA** (1973) The control of flux. *SEB Symposium* **27**: 65-104
- Kassab JY** (1988) *Applied Statistics*. University of Wales, Bangor.
- Keys AJ** (1986) Rubisco: its role in photorespiration. *Philos Trans R Soc, London. B* **313**, 325-336
- Kossman J, Sonnewald U, Willmitzer L** (1994) Reduction of the chloroplastic fructose-1,6-bisphosphatase in transgenic potato plants impairs photosynthesis and plant growth. *Plant J* **6**: 637-650
- Kimball BA** (1983) Carbon dioxide and agricultural yield: an assemblage and analysis of 430 prior observations. *Agron J* **75**: 779-788
- Kimball BA, Mauney JR, Radin JW, Nakayama FS, Idso SB, Hendrix DL** (1986) Effects of increasing atmospheric CO<sub>2</sub> on the growth, water relations and physiology of plants grown under optimal and limiting levels of water and nitrogen. In BR Strain, JD Cure, eds, *Direct effects of increasing carbon dioxide on vegetation*. United States Department of Energy, Office of Energy Research, Carbon Dioxide Research Division, Washington DC, pp 187-204



- Kimball BA, Pinter PJ Jr, Garcia RL, LaMorte RL, Wall GW, Hunsaker DJ, Wechsung G, Wechsung F, Kartschall T** (1995) Productivity and water use of wheat under free-air CO<sub>2</sub> enrichment. *Global Change Biol* **1**: 429-442
- Koide K, Ishihara K** (1992) Effects of ear removal on dry matter production and its partitioning in wheat and barley. *Jpn J Crop Sci* **61**(4): 651-658
- Krapp A, Hofmann B, Schäfer C, Stitt M** (1993) Regulation of the expression of *rbcS* and other photosynthetic genes by carbohydrates: a mechanism for the 'sink regulation' of photosynthesis. *Plant J* **3**: 817-828
- Krapp A, Quick WP, Stitt M** (1991) Ribulose-1,5-bisphosphate carboxylase-oxygenase, other photosynthetic enzymes and chlorophyll decrease when glucose is supplied to mature spinach leaves via the transpiration stream. *Planta* **186**: 58-69
- Krapp A, Stitt M** (1995) An evaluation of direct and indirect mechanisms for the sink-regulation of photosynthesis in spinach - changes in gas-exchange, carbohydrates, metabolites, enzyme-activities and steady-state transcript levels after cold-girdling source leaves. *Planta* **195**: 313-323
- Kriedemann PE, Wong SC** (1984) Growth responses and photosynthetic adaptation to carbon dioxide: Comparative behaviour in some C<sub>3</sub> species. *Adv Photosynth Res* **4**: 209-212
- Kuhlemeier C** (1992) Transcriptional and post-transcriptional regulation of gene expression in plants. *Plant Mol Biol* **19**: 1-14
- Labate CA, Leegood RC** (1988) Limitation of photosynthesis by changes in temperature. Factors affecting the response of carbon dioxide assimilation to temperature in barley leaves. *Planta* **173**: 519-527
- Lanzetta PA, Alvarez LJ, Reinach P, Candia O** (1979) An improved assay for nanomole amounts of inorganic phosphate. *Analytical Biochem* **100**: 95-97
- Lawlor DW** (1993) Photosynthesis: molecular, physiological and environmental processes. 2nd edn. Longman Scientific and Technical, Harlow
- Lawlor DW, Keys AJ** (1993) Understanding photosynthetic adaptation to changing climate. In L Fowden, J Stoddart, TA Mansfield, eds, *Plant adaptation to environmental stress*. Chapman and Hall, London, pp 85-106
- Lawlor DW, Konturri M, Young AT** (1989) Photosynthesis by flag leaves of wheat in relation to protein, ribulose bisphosphate carboxylase activity and nitrogen supply. *J Exp Bot* **40**: 43-52
- Lawlor DW, Mitchell RAC** (1991) The effects of increasing CO<sub>2</sub> on crop photosynthesis and productivity - a review of field studies. *Plant Cell Environ* **16**: 807-818

- Lawlor DW, Mitchell RAC, Franklin J, Mitchell VJ, Driscoll SP, Delgado E** (1993) Facility for studying the effects of elevated carbon-dioxide concentration and increased temperature on crops. *Plant Cell Environ* **16**: 603-608
- Leegood RC** (1993) The Calvin cycle and photorespiration. *In* PJ Lea, RC Leegood, eds, *Plant Biochemistry and Molecular Biology*. John Wiley and Sons Ltd, London, pp 27-45
- Leegood RC, Furbank RT** (1986) Stimulation of photosynthesis by 2% oxygen at low temperatures is restored by phosphate. *Planta* **168**: 84-93
- Leong T, Anderson JM** (1984) Adaptation of the thylakoid membranes of pea chloroplasts to light intensities. II. Regulation of electron transport capacities, electron carriers, coupling factor (CF<sub>1</sub>) activity and rates of photosynthesis. *Photosynth Res* **5**: 117-128
- Long SP** (1985) Leaf gas exchange. *In* J Barber, NR Baker, eds, *Photosynthetic mechanisms and the environment*. Elsevier, Amsterdam, pp 453-500
- Long SP** (1991) Modification of the response of photosynthetic productivity to rising temperature by atmospheric CO<sub>2</sub> concentrations: Has its importance been underestimated? *Plant Cell Environ* **14**: 729-740
- Long SP, Drake BG** (1992) Photosynthetic CO<sub>2</sub> assimilation and rising atmospheric CO<sub>2</sub> concentrations. *In* NR Baker, H Thomas, eds, *Crop photosynthesis: spatial and temporal determinants*. Elsevier Science Publishers, Amsterdam, pp 69-95
- Long SP, Hällgren JE** (1993) Measurement of CO<sub>2</sub> assimilation by plants in the field and the laboratory. *In* DO Hall, JMO Scurlock, HR Bolhar-Nordenkamp, RC Leegood, SP Long, eds, *Photosynthesis and production in a changing environment – a field and laboratory manual*. Chapman and Hall, London, pp 129-168
- Loreto F, Dimarco G, Tricoli D, Sharkey TD** (1994) Measurements of mesophyll conductance, photosynthetic electron-transport and alternative electron sinks of field grown wheat leaves. *Photosynth Res* **41**: 397-403
- Loreto F, Harley PC, Dimarco G, Sharkey TD** (1992) Estimation of mesophyll conductance to CO<sub>2</sub> flux by three different methods. *Plant Physiol* **98**: 1437-1443
- Machler F, Keys AJ, Cornelius MJ** (1980) Activation of ribulose biphosphate carboxylase purified from wheat leaves. *J Exp Bot* **31**: 7-14
- Makino A** (1994) Biochemistry of C<sub>3</sub>-photosynthesis in high CO<sub>2</sub>. *J Plant Res* **107**: 79-84

- Makino A, Mae T, Ohira K (1988)** Differences between wheat and rice in the enzymic properties of ribulose-1,5-bisphosphate carboxylase oxygenase and the relationship to photosynthetic gas-exchange. *Planta* **174**: 30-38
- Makino A, Nakano H, Mae T (1994)** Effects of growth temperature on the responses of ribulose-1,5-bisphosphate carboxylase, electron transport components, and sucrose synthesis enzymes to leaf nitrogen in rice, and their relationships to photosynthesis. *Plant Physiol* **105**: 1231-1238
- Makino A, Nakano H, Mae T, Shimada T, Yamamoto N (2000)** Photosynthesis, plant growth and N allocation in transgenic rice plants with decreased Rubisco under CO<sub>2</sub> enrichment. *J Exp Bot* **51**: 383-389
- Makino A, Osmond CB (1991)** Solubilisation of ribulose-1,5-bisphosphate carboxylase oxygenase from the membrane fraction of pea leaves. *Photosyn Res* **29**: 79-85
- Makino A, Sakashita H, Hidema J, Mae T, Ojima K, Osmond B (1992)** Distinctive responses of ribulose-1,5-bisphosphate carboxylase and carbonic anhydrase in wheat leaves to nitrogen nutrition and their possible relationships to CO<sub>2</sub>-transfer resistance. *Plant Physiol* **100**: 1737-1743
- Makino A, Sato T, Nakano H, Mae T (1997)** Leaf photosynthesis, plant growth and nitrogen allocation in rice under different irradiances. *Planta* **203**: 390-398
- Makino A, Shimada T, Takumi S, Kaneko K, Matsuoka M, Shimamoto K, Nakano H, Miyao-Tokutomi M, Mae T, Yamamoto N (1997)** Does decrease in ribulose-1,5-bisphosphate carboxylase by antisense *RbcS* lead to a higher N-use efficiency of photosynthesis under conditions of saturating CO<sub>2</sub> and light in rice plants? *Plant Physiol* **114**: 483-491
- Masle J, Hudson GS, Badger MR (1993)** Effects of ambient CO<sub>2</sub> concentration on growth and nitrogen use in transgenic tobacco (*Nicotiana tabacum*) plants transformed with an antisense gene to the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase. *Plant Physiol* **103**: 1075-1088
- McKee IF, Woodward FI (1994)** The effect of growth at elevated CO<sub>2</sub> concentrations on photosynthesis in wheat. *Plant Cell Environ* **17(7)**: 853-859
- McNeil PH, Walker DA (1981)** The effect of magnesium and other ions on the distribution of ribulose-1,5-bisphosphate carboxylase in chloroplast extracts. *Arch Biochem Biophys* **208**: 184-188
- Medlyn BE (1996)** The optimal allocation of nitrogen within the C<sub>3</sub> photosynthetic system at elevated CO<sub>2</sub>. *Aust J Plant Physiol* **23**: 593-603

- Mitchell RAC, Black CR, Burkart S, Burke JI, Donnelly A, de Temmerman L, Fangmeier A, Mulholland BJ, Theobald JC, van Oijen M (1999)** Photosynthetic responses in spring wheat grown under elevated CO<sub>2</sub> concentrations and stress conditions in the European, multiple-site experiment 'ESPACE-Wheat'. *Eur J Agron* **10**: 205-214
- Mitchell RAC, Gibbard CL, Mitchell VJ, Lawlor DW (1996)** Effects of shading in different developmental phases on biomass and grain yield of winter wheat at ambient and elevated CO<sub>2</sub>. *Plant Cell Environ* **19**: 615-621
- Mitchell RAC, Lawlor DW, Mitchell VJ, Gibbard CL, White EM, Porter JR (1995)** Effects of elevated CO<sub>2</sub> concentration and increased temperature on winter wheat: test of ARCWHEAT1 simulation model. *Plant Cell Environ* **18**: 736-748
- Mitchell RAC, Mitchell VJ, Driscoll SP, Franklin J, Lawlor DW (1993)** Effects of increased CO<sub>2</sub> concentration and temperature on growth and yield of winter wheat at two levels of nitrogen application. *Plant Cell Environ* **16**: 521-529
- Mitchell RAC, Theobald JC, Parry MAJ, Lawlor DW (2000)** Is there scope for improving balance between RuBP-regeneration and carboxylation capacities in wheat at elevated CO<sub>2</sub>? *J Exp Bot* **51**: 391-397
- Miziorko HM, Lorimer GH (1983)** Ribulose biphosphate carboxylase oxygenase. *Ann Rev Bioch* **52**: 507-535
- Moase EH, Green BR (1981)** Isolation and properties of chloroplast coupling factor from wheat. *Eur J Biochem* **119**: 145-150
- Morcuende R, Perez P, Martinez-Carrasco R, Martin Del Molino I, Sanchez De La Puente L (1996)** Long- and short-term effects of decreased sink demand on carbohydrate levels and photosynthesis in wheat leaves. *Plant Cell Environ* **19**: 1203-1209
- Mori H, Takabe T, Akazawa T (1994)** Loose association of ribulose-1,5-bisphosphate carboxylase/oxygenase with chloroplast thylakoid membranes. *Photosynth Res* **5**: 17-28
- Mott KA (1990)** Sensing of atmospheric CO<sub>2</sub> by plants. *Plant Cell Environ* **13**: 731-737
- Mousseau M, Saugier B (1992)** The direct effect of increased CO<sub>2</sub> on gas exchange and growth of forest tree species. *J Exp Bot* **43**: 1121-1130
- Mulholland BJ, Craigon J, Black CR, Colls JJ, Atherton J, Landon G (1997)** Effects of elevated carbon dioxide and ozone on the growth and yield of spring wheat (*Triticum aestivum* L.). *J Exp Bot* **48**: 113-122

- Nafziger ED, Koller HR (1976)** Influence of leaf starch concentration on CO<sub>2</sub> assimilation in soybean. *Plant Physiol* **57**: 560-563
- Nakano H, Makino A, Mae T (1995)** Effect of panicle removal on the photosynthetic characteristics of the flag leaf of rice plants during the ripening stage. *Plant Cell Physiol* **36**: 653-659
- Nakano H, Makino A, Mae T (1997)** The effect of elevated partial pressures of CO<sub>2</sub> on the relationship between photosynthetic capacity and N content in rice leaves. *Plant Physiol* **115**: 191-198
- Nie GY, Long SP, Garcia RL, Kimball BA, Lamorte RL, Pinter PJ Jr, Wall GW, Webber AN (1995a)** Effects of free-air CO<sub>2</sub> enrichment on the development of the photosynthetic apparatus in wheat, as indicated by changes in leaf proteins. *Plant Cell Environ* **18**: 855-864
- Nie GY, Hendrix DL, Webber AN, Kimball BA, Long SP (1995b)** Increased accumulation of carbohydrates and decreased photosynthetic gene transcript levels in wheat grown at an elevated CO<sub>2</sub> concentration in the Field. *Plant Physiol* **108**: 975-983
- Osborne CP, LaRoche J, Garcia RL, Kimball BA, Wall GW, Pinter PJ Jr, LaMorte RL, Hendrey GR, Long SP (1998)** Does leaf position within a canopy affect acclimation of photosynthesis to elevated CO<sub>2</sub>? *Plant Physiol* **117**: 1037-1045
- Parkinson KJ (1985)** A simple method for determining the boundary layer resistance in leaf cuvettes. *Plant Cell Environ* **8**: 223-226
- Parry MAJ, Andralojc PJ, Parmer S, Keys AJ, Habash D, Paul MJ, Alred R, Quick WP, Servaites JC (1997)** Regulation of Rubisco by inhibitors in the light. *Plant Cell Environ* **20**: 528-534
- Parry MAJ, Schmidt CNG, Cornelius MJ, Keys AJ, Millard BN, Gutteridge S (1985)** Stimulation of ribulose biphosphate carboxylase activity by inorganic orthophosphate without an increase in bound activating CO<sub>2</sub>: Co-operativity between the subunits of the enzyme. *J Exp Bot* **36**: 1396-1404
- Paul MJ, Driscoll SP (1997)** Sugar repression of photosynthesis: the role of carbohydrates in signalling nitrogen deficiency through source:sink imbalance. *Plant Cell Environ* **20**: 000-000
- Paul MJ, Knight JS, Habash D, Parry MAJ, Lawlor DW, Barnes SA, Loynes A, Gray JC (1995)** Reduction in phosphoribulokinase activity by antisense RNA in transgenic tobacco: effect on CO<sub>2</sub> assimilation and growth in low irradiance. *Plant J* **7**: 535-542
- Paul MJ, Lawlor DW, Driscoll SP (1990)** The effect of temperature on photosynthesis and carbon fluxes in sunflower and rape. *J Exp Bot* **226**: 547-555

- Peet MM, Huber SC, Patterson DT** (1986) Acclimation to high CO<sub>2</sub> in monoecious cucumbers. II. Carbon exchange rates, enzyme activities, and starch and nutrient concentrations. *Plant Physiol* **80**: 63-67
- Perchorowicz JT, Jensen RG** (1983) Photosynthesis and activation of ribulose biphosphate carboxylase in wheat seedlings. Regulation by CO<sub>2</sub> and O<sub>2</sub>. *Plant Physiol* **71**: 955-960
- Pick U, Bassilian S** (1981) Octyl glucoside stimulates a Mg<sup>++</sup>-specific ATPase activity in chloroplast CF<sub>1</sub>. *In* BR Selman, S Selman-Reimer, eds, *Energy coupling in photosynthesis*. Elsevier, Amsterdam, pp 251-260
- Pierpoint WS** (1996) The extraction of enzymes from plant tissues rich in phenolic compounds. *In* S Doonan, ed, *Methods in Molecular Biology: Protein Purification Protocols*, Vol 59. Humana Press Inc, Totowa, NJ, pp 69-80
- Pinter PJJ, Kimball BA, Garcia RL, all GW, Hunsaker DJ, LaMorte RL** (1996) Free-air CO<sub>2</sub> enrichment: responses of cotton and wheat crops. *In* GW Koch, HA Mooney, eds, *Carbon dioxide and terrestrial ecosystems*. Academic Press, San Diego, pp 215-249
- Portis AR, Salvucci ME, Ogren WL** (1986) Activation of ribulose biphosphate carboxylase/oxygenase at physiological CO<sub>2</sub> and ribulosebiphosphate concentrations by Rubisco activase. *Plant Physiol* **82**: 967-971
- Price GD, von Caemmerer S, Evans JR, Siebke K, Anderson JA, Badger MR** (1998) Photosynthesis is strongly reduced by antisense suppression of chloroplastic cytochrome *b/f* complex in transgenic tobacco. *Aust J Plant Physiol* **25**: 445-452
- Price GD, Yu JW, von Caemmerer S, Evans JR, Chow WS, Anderson JM, Hurry V, Badger MR** (1995a) Chloroplast cytochrome *b/f* and ATP-synthase complexes in tobacco: Transformation with antisense RNA against nuclear-encoded transcripts for the Rieske FeS and ATP8 polypeptides. *Aust J Plant Physiol* **22**: 285-297
- Price GD, Evans JR, von Caemmerer S, Kell P, Yu JW, Badger MR** (1995b) Specific reduction of chloroplast glyceraldehyde-3-phosphate dehydrogenase activity by antisense RNA reduces CO<sub>2</sub> assimilation via a reduction in ribulose biphosphate regeneration in transgenic tobacco plants. *Planta* **195**: 369-378
- Quick WP, Foyer CH** (1997) Preface. *In* WP Quick, CH Foyer, eds, *A molecular approach to primary metabolism in higher plants*. Taylor and Francis, Chichester, pp ix-x

- Quick WP, Schurr U, Scheibe R, Schulze ED, Rodermel SR, Bogorad L, Stitt M** (1991) Decreased ribulose-1,5-bisphosphate carboxylase-oxygenase in transgenic tobacco plants transformed with 'antisense' *rbcS*. I. Impact on photosynthesis in ambient growth conditions. *Planta* **183**: 542-554
- Rodermel SR, Abbott MS, Bogorad L** (1988) Nuclear-organelle interactions: nuclear antisense gene inhibits ribulose bisphosphate carboxylase enzyme levels in transformed tobacco plants. *Cell* **56**: 673-681
- Rogers GS, Milham PJ, Gillings M, Conroy JP** (1996) Sink strength may be the key to growth and nitrogen responses in N-deficient wheat at elevated CO<sub>2</sub>. *Aust J Plant Physiol* **23**: 253-264
- Rowland-Bamford AJ, Baker JT, Allen LH, Bowes G** (1991) Acclimation of rice to changing atmospheric carbon-dioxide concentration. *Plant Cell Environ* **14**: 557-583
- Rose M, Albige W, Entian KD** (1991) Glucose repression in *Saccharomyces cerevisiae* is directly associated with hexose phosphorylation by hexokinase PI and PII. *Eur J Biochem* **199**: 511-518
- Rufty TW** (1997) Probing the carbon and nitrogen interaction: a whole plant perspective. In WP Quick, CH Foyer, eds, *A molecular approach to primary metabolism in higher plants*. Taylor and Francis, Chichester, pp 255-275
- Sage RF** (1990) A model describing the regulation of ribulose-1,5-bisphosphate carboxylase, electron transport, and triose phosphate use in response to light intensity and CO<sub>2</sub> in C<sub>3</sub> plants. *Plant Physiol* **94**: 1728-1734
- Sage RF** (1994) Acclimation of photosynthesis to increasing atmospheric CO<sub>2</sub> – the gas-exchange perspective. *Photosynth Res* **39**: 351-368
- Sage RF, Reid CD** (1992) Photosynthetic acclimation to sub-ambient CO<sub>2</sub> (20 Pa) in the C<sub>3</sub> annual *Phaseolus vulgaris* L. *Photosynthetica* **27**: 605-617
- Sage RF, Sharkey TD** (1987) The effect of temperature on the occurrence of O<sub>2</sub> and CO<sub>2</sub> insensitive photosynthesis in field grown plants. *Plant Physiol* **84**: 658-664
- Sage RF, Reid CD, Moore BD, Seemann JR** (1993) Long-term kinetics of the light-dependent regulation of ribulose-1,5-bisphosphate carboxylase oxygenase activity in plants with and without 2-carboxyarabinitol 1-phosphate. *Planta* **191**: 222-230
- Sage RF, Sharkey TD, Seemann JR** (1988) The in-vivo response of ribulose-1,5-bisphosphate carboxylase activation state and the pool sizes of photosynthetic metabolites to elevated CO<sub>2</sub> in *Phaseolus vulgaris* L. *Planta* **174**: 407-416

- Sage RF, Sharkey TD, Seemann JR (1989) Acclimation of photosynthesis to elevated CO<sub>2</sub> in five C<sub>3</sub> species. *Plant Physiol* **89**: 590-596
- Sasek TW, DeLucia EH, Strain BR (1985) Reversibility of photosynthetic inhibition in cotton after long-term exposure to elevated CO<sub>2</sub> concentrations. *Plant Physiol* **78**: 619-622
- Sawada S, Enomoto S, Tozu T, Kasai M (1995) Regulation of the activity of ribulose-1,5-bisphosphate carboxylase in response to changes in the photosynthetic source-sink balance in intact soybean plants. *Plant Cell Physiol* **36**(4): 551-556
- Schäfer C, Simper H, Hofmann B (1992) Glucose feeding results in co-ordinated changes of chlorophyll content, ribulose-1,5-bisphosphate carboxylase-oxygenase activity and photosynthetic potential in photoautotrophic suspension cultured cells of *Chenopodium rubrum*. *Plant Cell Environ* **15**: 343-350
- Sharkey TD (1985) O<sub>2</sub> insensitive photosynthesis in C<sub>3</sub> plants. *Plant Physiol* **78**: 71-75
- Sharkey TD (1989) Evaluating the role of Rubisco regulation in photosynthesis of C<sub>3</sub> plants. *Phil Trans Royal Soc (London) B* **323**: 435-448
- Sharkey TD (1990) Feedback limitation of photosynthesis and the physiological role of ribulose bisphosphate carboxylase carbamylation. *Bot Mag Tokyo Special Issue* **2**: 87-105
- Sharkey TD, Kobza J, Seemann JR, Brown RH (1988) Reduced cytosolic fructose-1,6-bisphosphatase activity leads to loss of O<sub>2</sub> sensitivity in *Flaveria linearis* mutant. *Plant Physiol* **86**: 667-671
- Sharkey TD, Stitt M, Heineke D, Gerhardt R, Raschke K, Heldt HW (1986) Limitation of photosynthesis by carbon metabolism. II. O<sub>2</sub> insensitive CO<sub>2</sub> assimilation results from triose phosphate utilisation limitations. *Plant Physiol* **18**: 1123-1129
- Sheen J (1989) Metabolic repression of transcription in higher plants. *Plant Cell* **2**: 1027-1038
- Sheen J (1994) Feedback control of gene expression. *Photosynth Res* **39**: 427-438
- Shewry PR, Fido RJ (1996) Protein extraction from plant tissues. In S Doonan, ed, *Methods in Molecular Biology: Protein Purification Protocols*, Vol 59. Humana Press Inc, Totowa, NJ, pp 23-29
- Simpson RJ, Lambers H, Dalling MJ (1983) Nitrogen redistribution during grain growth in wheat (*Triticum aestivum* L.). *Plant Physiol* **71**: 7-14



- Sims DA, Luo Y, Seeman JR** (1998) Comparison of photosynthetic acclimation to elevated CO<sub>2</sub> and limited nitrogen supply in soybean. *Plant Cell Environ* **21**: 945-952
- Sionit N, Strain BR, Hellmers H** (1981) Effects of different concentrations of atmospheric CO<sub>2</sub> on growth and yield components of wheat. *J Agric Sci* **79**: 335-339
- Spencer W, Bowes G** (1986) Photosynthesis and growth of water hyacinth under CO<sub>2</sub> enrichment. *Plant Physiol* **82**: 528-533
- Stitt M** (1991) Rising CO<sub>2</sub> levels and their potential significance for carbon flow in photosynthetic cells. *Plant Cell Environ* **14**: 741-762
- Stitt M, Quick WP** (1989) Photosynthetic carbon partitioning: its regulation and possibilities for manipulation. *Physiol Plant* **77**: 633-641
- Stitt M, Quick WP, Schurr U, Schulze ED, Rodermeel SR, Bogorad L** (1991) Decreased ribulose-1,5-bisphosphate carboxylase-oxygenase in transgenic tobacco plants transformed with antisense *rbcS*. II. Flux control coefficients for photosynthesis in varying light, CO<sub>2</sub> and air humidity. *Planta* **183**: 555-566
- Stitt M, Schulze ED** (1994) Does Rubisco control the rate of photosynthesis and plant growth? An exercise in molecular eco-physiology. *Plant Cell Environ* **17**: 465-487
- Strotmann H, Hesse H, Edelmann K** (1973) Quantitative determination of coupling factor CF<sub>1</sub> of chloroplasts. *Biochemica Biophysica Acta* **314**: 202-210
- Teramura AH, Sullivan JH, Ziska LH** (1990) Interaction of elevated ultraviolet-B radiation and CO<sub>2</sub> on productivity and photosynthetic characteristics in wheat, rice, and soybean. *Plant Physiol* **94**: 470-475
- Terashima I, Evans JR** (1988) Effects of light and nitrogen on the organisation of the photosynthetic apparatus in spinach. *Plant Cell Physiol* **29**: 143-155
- Theobald JC, Mitchell RAC, Parry MAJ, Lawlor DW** (1998) Estimating the excess investment in ribulose-1,5-bisphosphate carboxylase/oxygenase in leaves of spring wheat grown under elevated CO<sub>2</sub>. *Plant Physiol* **118**: 945-955
- Tissue DT, Thomas RB, Strain BR** (1993) Long-term effects of elevated CO<sub>2</sub> and nutrients on photosynthesis and Rubisco in loblolly pine seedlings. *Plant Cell Environ* **16**(7): 859-865
- van Oijen M, Schapendonk AHCM, Jansen MJH, Pot CS, van Kleef J, Goudriaan J** (1998) Effects of elevated CO<sub>2</sub> on development and morphology of spring wheat grown in cooled and non-cooled open-top chambers. *Aust J Plant Physiol* **25**: 617-626

- van Oosten JJ, Besford RT (1994) Sugar feeding mimics effect of acclimation to high CO<sub>2</sub> – rapid down-regulation of Rubisco small subunit transcripts but not of the large subunit transcripts. *J Plant Physiol* **143**: 306-312
- van Oosten JJ, Besford RT (1995) Some relationships between the gas exchange, biochemistry and molecular biology of photosynthesis during leaf development of tomato plants after transfer to different carbon dioxide concentrations. *Plant Cell Environ* **18**: 1253-1266
- van Oosten JJ, Besford RT (1996) Acclimation of photosynthesis to elevated CO<sub>2</sub> through feedback-regulation of gene-expression – climate of opinion. *Photosynth Res* **48**: 353-365
- van Oosten JJ, Wilkins D, Besford RT (1995) Acclimation of tomato to different carbon dioxide concentrations. Relationships between biochemistry and gas exchange during leaf development. *New Phytol* **130**: 357-367
- von Caemmerer S, Evans JR (1991) Determination of the average partial pressure of CO<sub>2</sub> in chloroplasts from leaves of several C<sub>3</sub> plants. *Aust J Plant Physiol* **18**: 287-305
- von Caemmerer S, Evans JR, Hudson GS, Andrews TJ (1994) The kinetics of ribulose-1,5-bisphosphate carboxylase/oxygenase in vivo inferred from measurements of photosynthesis in leaves of transgenic tobacco. *Planta* **195**: 88-97
- von Caemmerer S, Farquhar GD (1981) Some relationships between the biochemistry of photosynthesis and the gas exchange of leaves. *Planta* **153**: 376-387
- von Schaewen A, Stitt M, Schmidt R, Sonnewald U, Willmitzer L (1990) Expression of yeast-derived invertase in the cell wall of tobacco and *Arabidopsis* plants leads to inhibition of sucrose export, accumulation of carbohydrate, and inhibition of photosynthesis, and strongly influences the growth and habitus of transgenic tobacco. *EMBO J* **9**: 3033-3044
- Vu JCV, Allen Jr LH, Bowes G (1987) Drought stress and elevated affects on soybean ribulose biphosphate carboxylase activity and canopy photosynthetic rates. *Plant Physiol* **83**: 573-578
- Wallsgrave RM, Keys AJ, Lea PJ, Mifflin BJ (1983) Photosynthesis, photorespiration and nitrogen metabolism. *Plant Cell Environ* **6**: 301-309
- \* Waters SP, Peoples MB, Simpson RJ, Dalling MJ (1980) Nitrogen redistribution during grain growth in wheat (*Triticum aestivum* L.). I. Peptide hydrolase activity and protein breakdown in the flag leaf, glumes and stem. *Planta* **148**: 424-428

- Watson RT, Meira-Filho LG, Sanhueza E, Janetos A** (1992) Sources and sinks. In JT Houghton, BA Challander, SK Varney, eds, Climate change 1992: the supplementary Report to the IPCC scientific assessment. Cambridge University Press, Cambridge, pp 25-46
- Webber AN, Nie GY, Long SP** (1994) Acclimation of photosynthetic proteins to rising atmospheric CO<sub>2</sub>. *Photosynth Res* **39**: 413-425
- Wittenbach VA** (1979) Ribulose biphosphate carboxylase and proteolytic activity in wheat leaves from anthesis through senescence. *Plant Physiol* **64**: 884-887
- Wong SC** (1979) Elevated atmospheric partial pressure of CO<sub>2</sub> and plant growth. I. Interactions of nitrogen nutrition and photosynthetic capacity in C<sub>3</sub> and C<sub>4</sub> plants. *Oecologia* **44**: 68-74
- Woodrow IW** (1994) Control of steady-state photosynthesis in sunflowers growing in enhanced CO<sub>2</sub>. *Plant Cell Environ* **17**: 277-286
- Woodrow IW, Berry JA** (1988) Enzymatic regulation of photosynthetic CO<sub>2</sub> fixation in C<sub>3</sub> plants. *Annu Rev Plant Physiol Plant Mol Biol* **39**: 533-594
- Yelle S, Beeson RC Jr, Trudel MJ, Gosselin A** (1989) Acclimation of two tomato species to high atmospheric CO<sub>2</sub>. II. Ribulose-1,5-bisphosphate carboxylase/oxygenase and phosphoenolpyruvate carboxylase. *Plant Physiol* **90**: 1473-1477
- Yokota A, Canvin DT** (1985) Ribulose biphosphate carboxylase oxygenase content determined with [C-14]carboxypentitol bisphosphate in plants and algae. *Plant Physiol* **77**: 735-739
- Younis HM, Winget GD, Racker E** (1977) Requirement of the  $\delta$  subunit of chloroplast coupling factor 1 for photophosphorylation. *J Biological Chem* **252**(5): 1814-1818
- Ziska LH, Hogan KP, Smith AP, Drake BG** (1991) Growth and photosynthetic response of nine tropical species with long-term exposure to elevated carbon dioxide. *Oecologia* **86**: 383-389
- Ziska LH, Teramura AH** (1992) CO<sub>2</sub> enhancement of growth and photosynthesis in rice (*Oryza sativa*). *Plant Physiol* **99**: 473-481



## Appendix A Publications arising from this study

**Theobald JC**, Mitchell RAC, Parry MAJ, Lawlor DW (1996) The interacting effects of nitrogen supply and CO<sub>2</sub> concentration on photosynthetic capacities in leaves of spring wheat. *Aspects of Applied Biology* **45**: 171-175

**Theobald JC**, Mitchell RAC, Parry MAJ, Lawlor DW (1998) Estimating the excess investment in ribulose-1,5-bisphosphate carboxylase/oxygenase in leaves of spring wheat grown under elevated CO<sub>2</sub>. *Plant Physiology* **118**: 945-955

Mitchell RAC, Black CR, Burkart S, Burke JI, Donnelly A, de Temmerman L, Fangmeier A, Mulholland BJ, **Theobald JC**, van Oijen M (1999) Photosynthetic responses in spring wheat grown under elevated CO<sub>2</sub> concentrations and stress conditions in the European, multiple-site experiment 'ESPACE-wheat'. *European Journal of Agronomy* **10**: 205-214

Mitchell RAC, **Theobald JC**, Parry MAJ, Lawlor DW (2000) Is there scope for improving balance between RuBP-regeneration and carboxylation capacities in wheat at elevated CO<sub>2</sub>? *Journal of Experimental Botany* **51**: 391-398